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High-Tech and Micropropagation VI

Edited by Y. P. S. Bajaj



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Biotechnology in Agriculture and Forestry 40

High-Tech and Micropropagation VI

Edited by Y.P.S. Bajaj

With 168 Figures and 84 Tables



Springer

Professor Dr. Y.P.S. BAJAJ

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Dedicated to

Dr. Leo Dionne

with whom I had the pleasure of working

at the Canada Department of Agriculture,

Fredericton, New Brunswick (Canada) during 1965–1967

Preface

Micropropagation of plants is a multibillion dollar industry being practiced in hundreds of small and large nurseries and commercial laboratories throughout the world. At present, it is the only component of plant biotechnology which has been commercially exploited on such a large scale, especially for the production of ornamentals. Now micropropagation of trees and medicinal plants has also assumed great importance. With recent progress made in the propagation of fruit and forest trees, and the immediate need for afforestation and planting of orchards, propagules and plantlets are required quickly and in large numbers. Taking these points into consideration *High-Tech and Micropropagation I, II, III, and IV* were published in 1991 and 1992. The present two volumes, *High-Tech and Micropropagation V and VI*, comprise 51 chapters contributed by international experts from 24 countries.

High-Tech and Micropropagation V comprises 24 chapters arranged into the following three sections:

- I. Vegetables and fruits (garlic, *Amaranthus*, *Brassica oleracea*, pepper, watermelon, cassava, banana, *Myrtus communis*, passionfruit, *Polymnia sonchifolia*, pepino, and spinach)
- II. Grasses (bamboos, *Caustis dioica*, *Dendrocalamus*, *Miscanthus x giganteus*, sugarcane)
- III. Trees (*Aegle marmelos*, *Eucalyptus*, *Fraxinus excelsior*, *Juglans cinerea*, *Pinus virginiana*, *Prosopis*, and *Ulmus* species)

High-Tech and Micropropagation VI comprises 27 chapters arranged in two sections:

- I. Ornamental and aromatic plants (*Amaryllis*, *Anthurium*, *Blandfordia*, bromeliads, *Campanula*, *Coleus*, *Ctenanthe*, *Cyclamen*, *Daphne*, *Dracaena*, *Gerbera*, *Helianthemum*, *Hippeastrum*, *Leucojum*, *Mammillaria*, *Maranta*, *Mediocactus*, *Mussaenda*, *Narcissus*, *Otacanthus*, ponytail palm, *Prunus tenella*, *Spiranthes*, and *Zinnia*)
- II. Medicinal and miscellaneous plants (*Duboisia*, *Matricaria*, *Sideritis*, *Dictamnus albus*, *Simmondsia chinensis*)

These books will be of use not only to advanced students, research workers, and teachers in the field of horticulture, forestry, pharmacy, tissue culture, and plant biotechnology in general, but also to individuals interested in industrial micropropagation.

New Delhi, February 1997

Professor Dr. Y.P.S. BAJAJ
Series Editor

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Section I

Ornamental, and Aromatic Plants

I.1 Micropagation of Amaryllis (*Hippeastrum hybridum*)

M.H. DE BRUYN¹

1 Introduction

1.1 Botany, Distribution, and Importance

The genus *Hippeastrum* of the Amaryllidaceae family consists of 75 species which, apart from one West African species, all originated in South America (Bailey Hortorium Staff 1976). *Hippeastrum* is commonly known as the giant amaryllis, Barbados lily, fire lily or the Royal Dutch amaryllis (Macoboy 1969).

Amaryllis is a perennial bulbous plant that normally grows from October to March in the Southern Hemisphere, although the plants can be cultivated all year round in glasshouses (Opred 1989). A plant usually produces three to six glossy strap-like leaves which are approximately 600 mm long and 50 mm wide. The flowers appear at the same time as the leaves, and one plant can produce one or two inflorescences. An inflorescence can generate two to five (mostly four) big trumpet-shaped flowers (Fig. 1). The scape is usually hollow and can grow up to 550 mm (Rockwell and Grayson 1977). The colour of the flowers can vary from pure white to brilliant red, with many colour and striped variations in between. The flowers are zygomorphic and are almost 200 mm in diameter. The seeds are flattened, with black papery wings (Bailey Hortorium Staff 1976).

The first plants were brought to Europe in 1700 and the first interspecies crossings were made in 1799 when *H. reginae* was crossed with *H. vittatum* (Anonymous 1981; Meerow et al. 1991). Other species that played an important part in the makeup of the hybrids known today are *H. aulicum*, *H. reticulatum*, *H. rutilum* and *H. striatum*, which all came from Brazil (Bailey Hortorium Staff 1976) and *H. psittacinum*, *H. leopoldii* and *H. pardinum*, which originated in Peru (Meerow et al. 1991). *Hippeastrum johnsonii* and *H. ackermanii* also originated in South America and were incorporated in the breeding programme at a later stage (Anonymous 1981; Meerow et al. 1991). *H. vittatum* was used in several of the crossings and was mainly responsible for the varieties with the striped perigon leaves (Eliovson 1967; Anonymous 1981), while *H. leopoldii* and *H. pardinum* were used for the breeding of plants with bigger flowers. Recently, mainly hybrids have been used in the breeding programmes.

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Fig. 1. The flower of amaryllis (*Hippeastrum hybrideum*)

Amaryllis is a well-known pot and garden plant, as well as a cut flower, and is planted on a commercial scale in The Netherlands, South Africa and the United States of America, especially in the state of Florida (Meerow et al. 1991). The Netherlands is the largest producer of amaryllis in the world, and much time and money are spent on the evaluation and improvement of the crop (De Geler 1990; De Geler and Ettema 1990). In The Netherlands, 103 ha of glasshouse space were used to cultivate amaryllis, of which approximately a third was for cut flowers and the rest for flower bulb production (Opred 1989). The production of amaryllis amounted to approximately 54 million flowers in 1991 (Boersma 1992). The flowers are produced mostly for the export market and nearly 50% of the flowers that are produced in The Netherlands are exported, mostly to European countries (Boersma 1992). In South Africa, the bulbs have been cultivated since 1950 (Barnhoom 1991), and well-known cultivars such as Zanzibar, Safari and Tangerine and more lately Sun Dance have been produced. About 24 cultivars are freely available in the trade market (Van der Wiel 1988). The production in Florida has decreased lately due to plant disease and market competition, but there are still some workers in the USA, like De Hertog and Tilley (1991), doing research on the effect of different planting media on flowering of amaryllis bulbs.

1.2 Conventional Propagation and Need for Micropropagation

Bulbous plants are usually propagated by seed or by dividing the clumps of offset bulbs that had been produced during the last growth period, but methods like chipping and scaling of the bulb can also be used (Rees 1992). The

method of scaling consists of removing each scale from the mother bulb, dipping it in a disinfectant, and planting it in a sterile rooting medium. A variation on this technique is twinscaling, where the scales are separated in pairs linked by a piece of basal plate. This technique usually provides a higher yield than single scales (Huang et al. 1990a; Rees 1992). Chipping, on the other hand, is less damaging for the plant and can be mechanised for use on large numbers of bulbs. The bulbs should preferably be round and 10–12 cm in diameter. The bulbs are cleaned of all dry outer scales and are surface sterilized, after which the bulb is divided into 16 radial segments or chips, each with a piece of basal plate. These chips are then dipped into a antifungal solution and planted in damp vermiculite (Rees 1992). New bulblets are then formed between the fleshy scales.

Although *in vivo* methods can be used for the multiplication of amaryllis as described above, there are certain advantages of *in vitro* propagation. The first advantage would be a higher multiplication rate, especially the secondary multiplication. The whole multiplication process is performed under aseptic conditions which results in a higher survival rate of the explants (pieces of bulb tissue placed on growth medium), and the plants are kept disease-free. It is also possible to divide the small plantlets that formed *in vitro*, for a secondary multiplication.

2 In Vitro Culture Studies and Micropagation

2.1 General Account

The disinfection of bulbs is usually the most difficult part, as this subterrestrial explant material is in such close contact with a wide variety of soil microorganisms. The bulbs are usually rinsed in ethanol before surface sterilization but different workers use different concentrations. Bapat and Narayanaswamy (1976) used full strength ethanol while Hussey (1980) used 90% ethanol and Huang et al. (1990b) preferred to use 70% ethanol. For the disinfection process most authors use sodium hypochloride (NaOCl) but the time of exposure and the concentration of the solution differs. Huang et al. (1990b) used a 10% NaOCl solution for a period of 10 min for the disinfection of amaryllis bulbs while Jana (1981) used a 3 to 5% NaOCl solution for 15 min, and De Bruyn (1990) a 2% NaOCl solution for 60 min. Mii et al. (1974) used a 1% NaOCl solution for 8 min, and after the explants were dissected, they were disinfected for a further period of 2 min in the NaOCl solution. Other disinfectants that have also been used are mercuric chloride (Bapat and Narayanaswamy 1976; 0.1% for 15 min) or calcium chloride (Jana 1981; 3–5% for 15 min; Seabrook and Cumming 1977, 8% for 15 min). To enhance the effect of the disinfection some authors use a detergent like Tween 20 or 7X. A hot water treatment can also be used to enhance the disinfection (De Bruyn et al. 1992).

Different parts of the plant have been used as explant material. Mii et al. (1974), Pajerski and Ascher (1977) and Seabrook and Cumming (1977) used bulb scales without any part of the basal plate, while Jana (1981) used bulb scales with a piece of basal plate as explant material. Hussey (1975), De Bruyn (1990) and Huang et al. (1990a,b) used twinscales, and Bapat and Narayanaswamy (1976) used the basal plate without any scale tissue. Some authors have also used the peduncle and floral parts like the anthers, style filament, ovule and perianth as explant material (Bapat and Narayanaswamy 1976; Pajerski and Ascher, 1977; Seabrook and Cumming 1977; De Bruyn 1990; Pierik et al. 1990; Pierik 1991). Mujib et al. (1991) first initiated callus from immature flower buds before regenerating plantlets. Small bulbs formed in vitro are also used as explant material (Hussey 1980; De Bruyn 1990; Pierik et al. 1990).

The mixture of inorganic salts as compiled by Murashige and Skoog (1962) is the nutrient medium (MS) that is used most often (Hussey 1975; Bapat and Narayanaswamy 1976; Seabrook and Cumming 1977; Hussey 1980; De Bruyn 1990). In some instances, a half-strength solution of these salts is used (Hussey 1980) or a different mixture of vitamins is used (Bapat and Narayanaswamy 1976). Some authors use a completely different combination of inorganic salts, like Jana (1981), used a modified nutrient medium of Knudson (1946), and Blakesly and Constantine (1992), used the medium of Linsmaier and Skoog (1965).

A variety of plant growth regulators are used in combination with the inorganic salts, but the combination of benzyladenine (BA) and naphthaleneacetic acid (NAA) is most frequent (Seabrook and Cumming 1977; Hussey 1980). Other growth regulators that have also been used are kinetin (Mii et al. 1974), 2,4-dichlorophenoxy acetic acid (2,4-D; Bapat and Narayanaswamy 1976; Seabrook and Cumming 1977), indole acetic acid (IAA) or zeatin (Huang et al. 1990b).

Most authors use 2 to 3% sucrose as a source of carbohydrate and the media are solidified with 0.6 to 1% agar (Mii et al. 1974; Hussey 1975; Bapat and Narayanaswamy 1976; Hussey 1980; Huang et al. 1990b). Pierik (1991) did a study on the type of agar that should be used in the growth media for the culturing of different plants.

The cultures are incubated at 20 to 25°C (Seabrook and Cumming 1977; Jana 1981), in either continuous light (Mii et al. 1974; Bapat and Narayanaswamy 1976; Hussey 1980; Huang et al. 1990b; Pierik et al. 1990) or a light cycle of 16h light 8h dark (Seabrook and Cumming 1977).

2.2 Micropagation Techniques

The recommended technique does not differ much from the methods used by others, but there are variations that could make a difference in the multiplication rate.

2.2.1 Disinfection

For the disinfection of mature bulbs, the bulbs are harvested and rinsed under tap water to remove excess soil and dust. The best time of the year to harvest the bulbs is at the beginning of the growth cycle (De Bruyn 1990). The leaves and roots are removed without removing too much of the basal plate. The bulbs are washed and cleaned thoroughly and all the dried outer scales removed. They are then disinfected by gentle shaking in 96% ethanol for 2 min, after which the bulbs are transferred to a 2% NaOCl (+ 7X) solution for 30–45 min, depending on the size of the bulb. The bulbs are then rinsed three times in sterile distilled water.

If difficulty is encountered in securing a successful disinfection process, a hot water treatment with wide-spectrum fungicides can be included in the procedure, just before the bulbs are rinsed in ethanol. During this treatment, the bulbs are soaked in a solution of Benomyl (2 g/l) and Folpet (4 g/l) at 50 °C for a 30 min, before being rinsed in ethanol (De Bruyn et al. 1992). For small bulbs this treatment can be too harsh, and a longer exposure to the fungicide at a lower temperature is recommended (45 min at room temperature).

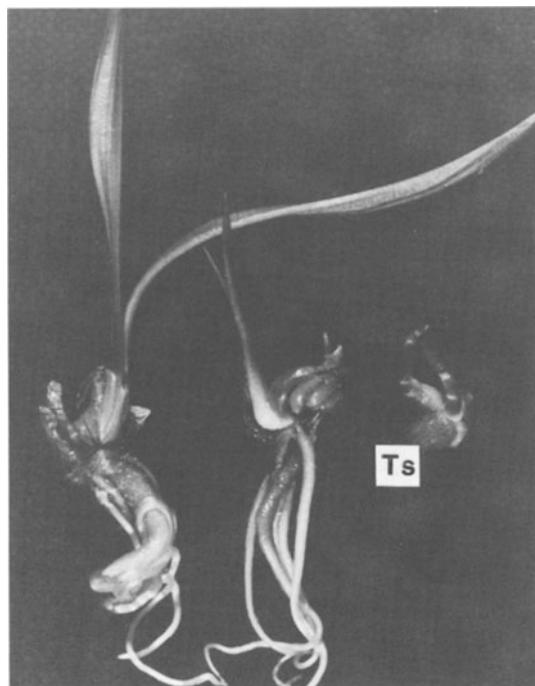


Fig. 2. Plantlets formed by twinscales of amaryllis cultured on MS medium containing 5 mg/l BA and 0.1 mg/l NAA. *Ts* twinscale. (De Bruyn 1990)

2.2.2 Explant Material

Different plant parts such as bulb scales, twinscales and young scapes can be used as explant material. The best time of the year to use bulbs for explant material is at the beginning of the growth phase as soon as the leaves start to emerge (De Bruyn 1990). The bulb scales can be used as explant material, but they produce much less plantlets than the twinscales, and some explants form only callus (De Bruyn 1990). A twinscale consists of two bulb scales (25 mm long \times 10 mm wide) attached to each other by a piece of basal plate tissue (4 mm long; Fig. 2). There is no noticeable difference between the number of plantlets formed by the twinscales on the inside of the bulb and those on the outside, which means that all the twinscales can be used with a similar multiplication rate. From an anatomical study it became clear that the bulblets are produced from scale tissue that is located very close to the basal plate on the abaxial side of the scale (Fig. 3; De Bruyn 1990; Huang et al. 1990b). Most of the explants formed new plantlets between the two scales, but some plants were also produced on the exposed sides of the twinscale. Meristematic activity is already noticeable within 1 week after the explants are

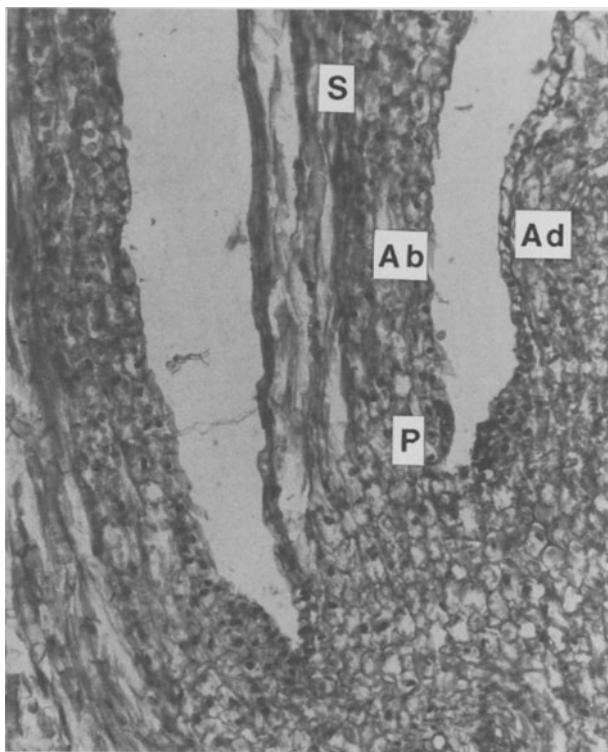


Fig. 3. Twinscales of amaryllis formed a protuberance on the abaxial side of the inner scale after 2 weeks in culture. *S* Bulb scale; *P* protuberance; *Ad* adaxial side; *Ab* abaxial side. (De Bruyn 1990)

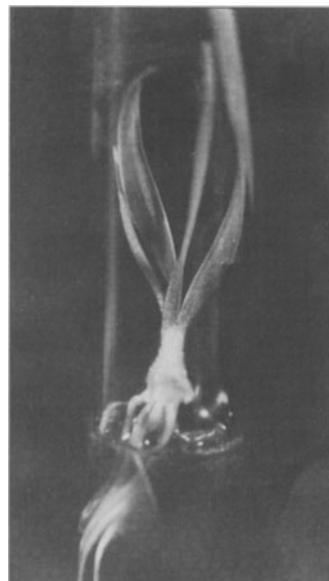
inoculated on the growth medium, and after 2 weeks a protuberance starts to form (Fig. 3). Differentiation takes place after approximately 3 weeks. The orientation of the twinscales on the medium does not make a significant difference to the number of plantlets that are formed, but the twinscales that are placed with their scales into the medium tend to produce plantlets in an upside-down position and the leaves then push the plantlets out of the growth medium, which is detrimental for the development of the plantlet. It is therefore better to orientate the explant with the basal plate into the medium. The plantlets that are formed in vitro can be divided further to facilitate mass propagation (Fig. 4). The plantlets are divided into four equal parts, each with a piece of basal plate tissue, which is transferred to new medium.

The advantage of using young scape tissue as explant material is that the mother bulb does not have to be destroyed to multiply the plant, but the problem is that this material is not always readily available. The scapes should be used while they are still in an actively growing phase (between 30 and 75 mm). The scapes are dissected into 5-mm-thick disks and are placed with either their proximal or distal side on the medium. Most plantlets are formed when the discs from the proximal end of the scape are placed with the proximal side onto the medium (De Bruyn 1990). The last disc of the scape forms new plantlets between the basis of the flower stalks on the distal side of the disc when the disc is placed with its proximal end on the medium.

2.2.3 Culture Medium and Incubation

The explants are cultivated on the basic nutrient MS medium that is supplemented with 5 mg/l BA and 0.1 mg/l NAA. A sucrose concentration of 2 or 3%

Fig. 4. An in vitro plantlet of *Hippeastrum hybridum* with a diameter of 6 mm that can be used for secondary multiplication. (De Bruyn 1990)



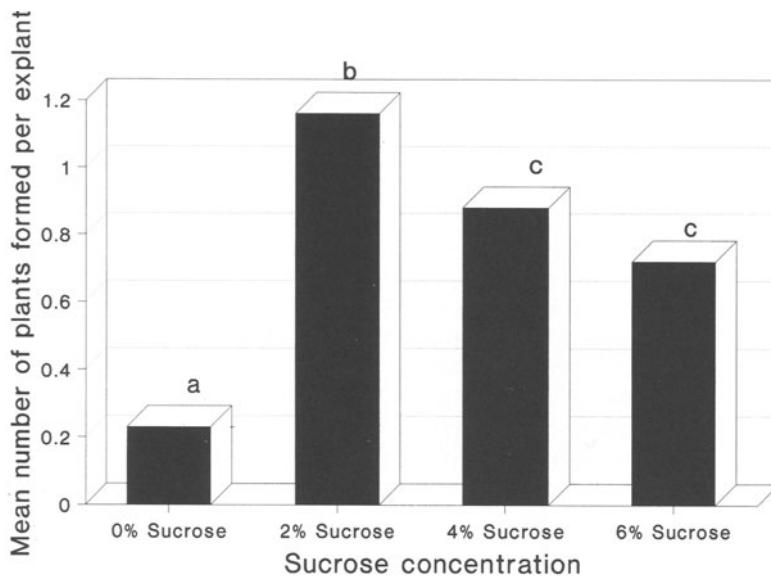


Fig. 5. Number of plantlets formed by *Hippeastrum hybrideum* twinscale explants on media containing different sucrose levels. Treatments with different symbols differ significantly at the 5% level

is used, as a higher concentration has a negative effect on the number of bulblets formed by twinscale explants (Fig. 5). The scape explants produce more plantlets on a growth medium with a higher sucrose content (9% sucrose), but the growth rate of these plantlets is slower than those cultured on a medium with only 3% sucrose (De Bruyn 1990). The pH of the medium is set to 5.75 and the medium solidified with 0.2% Gelrite before autoclaving at 120°C and 115 kPa for 20 min.

In comparison with the plantlets that are cultured on a solidified medium, plantlets that are cultured on liquid growth medium tend to show much vitrification and do not grow as vigorously (De Bruyn 1990).

The cultures are incubated at a constant temperature of 24 °C (± 1 °C) and a photoperiod of 16 h light and 8 h dark. The light is provided by cool white fluorescent light tubes at $25 \mu\text{E}/\text{m}^2/\text{s}$.

3 Summary and Conclusions

The in vitro propagation of amaryllis bulbs is feasible and is being used to some extent by commercial laboratories. The biggest advantage of this procedure is that disease-free, clonal plants can be produced at a rate that is much faster than is possible with traditional horticultural methods. The best explants for establishing in vitro cultures are twinscales or young scapes (between 30

Table 1. Summary of the work done on micropagation of amaryllis

Type of explant	Medium	Response/Remarks	Reference
Bulb scales without basal plate	MS; NAA and kinetin	Successful results were obtained in September, but not at other times of the year	Mii et al. (1974)
Twinscales	MS; NAA (2 mg/l)	Adventitious bulbils are formed from the tissue layer at the junction of the bulb scales and the basal plate	Hussey (1975)
Basal plate without scale tissue; peduncle and floral parts	MS; vitamins as formulated by Lin and Staba (1961); with or without auxins	Shoot bud regeneration was hastened by the sequential withdrawal of auxin from an auxin-enriched medium on which the callus had been growing	Bapat and Narayanaswamy (1976)
Bulb scales without basal plate; peduncle and floral parts	Linsmaier and Skoog; NAA (0.3 mg/l)	The most responsive tissue was from scales and peduncles	Pajerski and Ascher (1977)
Bulb scales without basal plate; peduncle and floral parts	MS; 2,4-D and BA	Shoots placed on media containing 2–4 mg/l BA and 0.03–0.5 mg/l NAA show irregular bursts of proliferation but this does not happen consistently	Seabrook and Cumming (1977)
Leaf base and stem tissue; in vitro-formed bulblets	MS (full- or half-strength); NAA (0.5–8 mg/l)		Hussey (1980)
Bulb scales with a piece of basal plate	Knudson (modified)		Jana (1981)
Twinscales; peduncle and floral parts; in vitro-formed bulblets	MS; BA (5 mg/l) and NAA (0.1 mg/l)	Twinscales and in vitro-formed bulblets gave the best results for propagation of bulbs	De Bruyn (1990)
Twinscales and single scales	MS; no growth hormones or zeatin (1 mg/l)	Bulbils are regenerated directly by twinscales while single scales form protocorm-like bodies without vascular connection to the scale tissue	Huang et al. (1990)
In vitro-formed bulblets; peduncle and floral parts	MS	Plants need a relatively high temperature, continuous darkness, a high sucrose concentration in the medium and an auxin and cytokinin supply	Pierik et al. (1990)
Floral stems	MS; BA (2 mg/l) and NAA (0.2–0.4 mg/l)	25°C and continuous darkness	Pierik (1991)
Callus from immature flower buds	MS; BA (0.5 mg/l) and IBA		Mujib et al. (1991)

and 75 mm long). The bulbs are surface sterilized and dissected into radial segments, each with a piece of basal plate. The radial segments are divided into twinscales and are also transferred to growth medium. The nutrient medium consists of MS medium containing 5 mg/l BA, 0.1 mg/l NAA, 3% sucrose and 2 g/l Gelrite (pH 5.75). The cultures are incubated at 24°C under a photoperiod of 16 h light and 8 h dark. If a young inflorescence is present in the bulb, it should be removed, cut into discs and inoculated on growth medium. The small bulbs that are produced in vitro can also be used for further multiplication. Although the in vitro propagation of amaryllis is not more cost-effective than conventional propagation techniques, this provides a tool for the rapid propagation of plant material when a limited number of mother plants is available, such as new hybrids. *Hippeastrum* is a bulbous plant, which makes it relatively easy to transfer these plantlets to pots and eventually to the field.

4 Protocol

1. Wash and clean the bulbs.
2. Remove the leaves, roots and dry outer tunics.
3. Treat the bulbs with a fungicide solution (Benomyl 2 g/l and Folpet 4 g/l) at 50°C for a period of 30 min.
4. Disinfect bulbs in 2% NaOCl for 45 min and rinse three times in sterile distilled water.
5. Dissect bulbs and divide into twinscales.
6. Transfer the explants to MS medium containing 5 mg/l BA and 0.1 mg/l NAA and 3% sucrose.
7. Incubate the cultures at 24°C and a photoperiod of 16 h light and 8 h dark.

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I.2 Micropropagation of *Anthurium*

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1 Introduction

1.1 Distribution, Botany, and Importance of *Anthurium*

Within the family Araceae, *Anthurium* is the largest, most morphologically diverse and complex genus, consisting of approximately 1000 species. Native to Central and South America, members of *Anthurium* are found at elevations ranging from sea level to 3000 m, most commonly in cloud forests at 1500 m (Croat 1986). Plants of this herbaceous perennial monocot are terrestrial or epiphytic. Typical of the aroids is the spadix, consisting of a multitude of unobtrusive true flowers supported by a fleshy axil. The protogynous nature of the bisexual flowers in *Anthurium* favors cross-pollination. The commercial flower is a combination of the spadix and a colorful modified leaf, termed spathe. Attractive foliage of some species makes anthuriums also suitable for leaf harvest and cultivation as a potted plant.

Commercial production has focused on plants derived from two major species, *Anthurium andraeanum* Linden ex. Andre and *A. scherzerianum* Schott. (Fig. 1A,B). The majority of the plants used in the cut flower industry are thought to be hybrids of *A. andraeanum* and other species (Madison 1980), and will be referred to as *A. andraeanum* Hort. Main production areas are Hawaii and The Netherlands, with additional production in other tropical and subtropical regions. The 1991, combined Dutch auctions ranked anthurium 14th of all cut flower sales, with over 20 million stems sold for approximately \$21.5 million (International Floriculture Quarterly Report 1992). Estimates for Dutch auction anthurium sales in 1993 are approximately 37 million stems (International Floriculture Quarterly Report 1994). In Hawaii, anthurium is one of the top cut flowers, with a 1993 farmgate value of sales of \$7.5 million for 10.6 million stems sold (Hawaii Agricultural Statistics Service 1994). *A. scherzerianum* is sold as a flowering potted plant, with main production areas located in Europe. Global production of anthurium hybrids as potted plants has recently increased.

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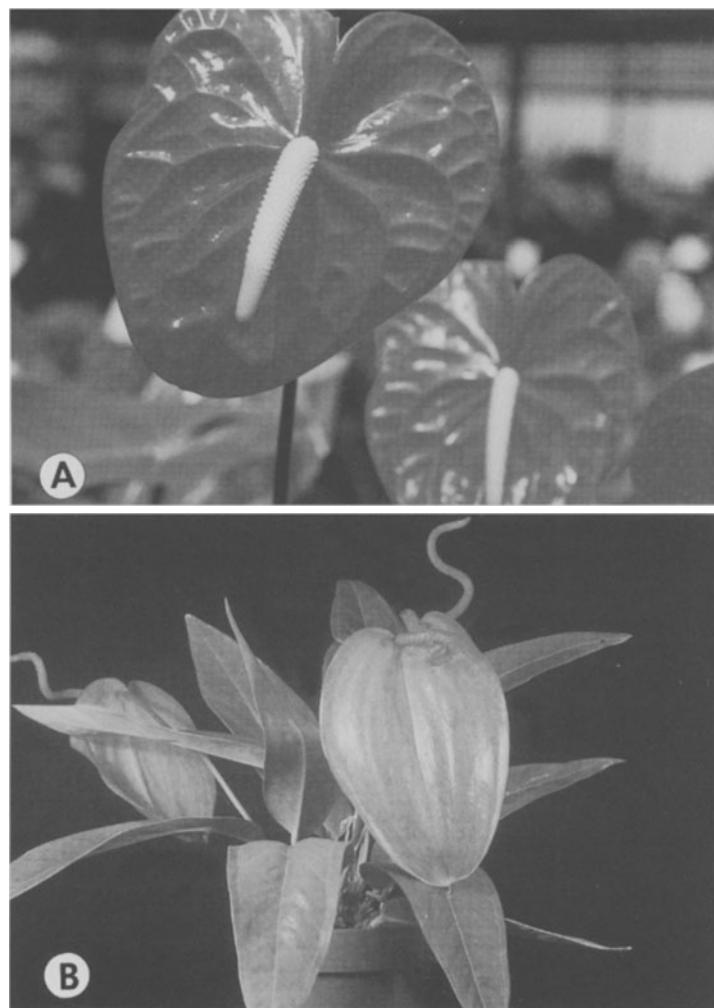


Fig. 1. A *Anthurium andraeanum* Hort., cut flower cultivar Nitta. **B** *Anthurium scherzerianum* used in flowering potted plant production. (Photos courtesy H. Kamemoto)

1.2 Common Propagation Practices and Need for Microppropagation

Conventional propagation relies upon divisions, cuttings, and in vitro methods. Seeds are less commonly used for propagation, as they may produce heterogeneous populations varying in flower color, size, and form.

Division relies on lateral shoots arising from the basal stem portion of the anthurium plant. Some cultivars produce lateral shoots easily while others produce very few. Time until first harvestable flower depends upon initial size of the division, but is generally in terms of months rather than years. Plant growth regulators have been used to stimulate lateral shoot development

within 4 to 6 months after application. A foliar spray of 1000mg/l benzyladenine (BA) applied to intact Ozaki plants resulted in 3.6 lateral shoots per plant, with zero lateral shoots formed on unsprayed plants (Higaki and Rasmussen 1979). Removal of the apical portion of juvenile Mauna Kea plants followed by a 500mg/l GA₃ spray increased shoot production from 3.3 shoots (without spray) to an average of 5.8 shoots per sprayed plant (Imamura and Higaki 1988).

Top cuttings, consisting of the uppermost stem with two or three leaves, are removed from plants and rooted in a well-aerated medium. Roots develop within 2 to 3 weeks, with the first flower produced in approximately 6 months. Removal of the top cutting stimulates development of lateral shoots on the mother plant. Basal cuttings, consisting of one or two leafless nodal sections and placed horizontally on medium, produce plants from each node. Although more plants may be generated by basal, rather than top, cuttings, plants take longer to develop and often require 2 to 3 years to reach full production.

In vitro propagation is another method commonly used in anthurium propagation. Plants may be obtained directly from excised apical and lateral buds or indirectly through the differentiation of callus induced from leaf, spathe, and spadix explants.

Rapid clonal propagation is an important use of anthurium tissue culture. In The Netherlands, anthuriums are almost exclusively propagated by culture in vitro (van Doesburg 1991). As recalcitrant genotypes continue to hinder micropropagation of some cultivars, additional in vitro studies should be undertaken.

Disease elimination should be addressed in the propagation of anthuriums. The bacterial blight caused by *Xanthomonas campestris* pv. *dieffenbachiae* has been a problem in Hawaii for a decade, and occurs in other anthurium-producing countries such as the Philippines, Jamaica, Tahiti, and Trinidad. *Xanthomonas campestris* pv. *dieffenbachiae* may be present in callus and stage II plantlets without visible symptoms, and will not cause medium turbidity in MS-based media that lack coconut water (Norman and Alvarez 1994). Efforts are underway to develop an indexing and certification program for micropropagated anthuriums (Tanabe et al. 1992; Fernandez et al. 1992).

Due to the long breeding cycle of anthuriums, in which the development of a new cultivar may take 8 to 10 years, genetic engineering as a viable breeding aid is currently being investigated at the University of Hawaii for resistance to bacterial blight (Kuehnle et al. 1992b).

2 Review of In Vitro Studies

Early work on anthurium tissue culture described callus proliferation and subsequent plantlet formation from seeds and young leaf tissue of mature anthurium plants (Pierik et al. 1974). Following this pioneering work, many studies have been conducted for several species and hybrids (reviewed by Geier 1990; Table 1). Different culture media containing modifications of

Table 1. Summary of microppropagation studies in *Anthurium*. (See also Geier 1990)^a

Anthurium species/hybrids	Explant material	Observations/remarks	Reference
<i>A. andraeanum</i>	In vitro plant	Acclimatization of rooted plants	Imamura and Higaki (1981)
<i>A. scherzerianum</i>	Spadix fragments	Ploidy variation in callus and regenerated plants	Geier (1982,1988)
<i>A. andraeanum</i>	Seed	Multiple plant formation from seeds germinated in vitro	Tanabe et al. (1989)
<i>A. andraeanum</i>	Lamina	Callus and plant regeneration on medium with BA and/or 2,4-D	Lightbourn and Devi Prasad (1990)
<i>A. andraeanum</i>	In vitro plant	Certification and indexing procedure for detection of <i>Xanthamonas campestris</i> pv. <i>diffenbachiae</i>	Fernandez et al. (1992) Tanabe et al. (1992)
<i>A. andraeanum</i>	Etiolated shoot	Protoplast obtained but sustained division not observed	Kuehnle and Nan (1991)
<i>A. andraeanum</i>	Lamina and petiole	Shoot and roots from caulogenic callus, genotype effect on regeneration	Kuehnle and Sugii (1991)
<i>A. andraeanum</i>	Lamina and spadix	Spadix explants produced more callus formation and plant regeneration than lamina, with greater ploidy uniformity; some aneuploids were obtained	Singh and Sangama (1991)
<i>A. andraeanum</i>	In vitro plant	Stage II and stage III plant acclimatization	Tanabe (1991)
<i>A. andraeanum</i>	Bud	Effect of genotype on time to first leaf formation	Tanabe et al. (1991)
<i>A. andraeanum</i> <i>A. lindenianum</i> <i>A. amnicola</i> <i>A. kamemotoanum</i>	In vitro lamina	Somatic embryos obtained and produced single plants or multiple plant clumps	Kuehnle et al. (1992)
<i>A. scherzerianum</i>	Lamina and petiole	Caulogenic callus and plant regeneration	Liu and Xu (1992)
<i>A. andraeanum</i>	Bud	Surface disinfection of buds	Tanabe and Matsumoto (1992)
<i>A. cubense</i>	Lamina and petiole	Examined leaf stage, and medium NH_4NO_3 and 2,4-D on callus formation	Warner et al. (1993)
<i>A. andraeanum</i>	Lamina, petiole, callus and plantlets	<i>Xanthamonas campestris</i> pv. <i>diffenbachiae</i> may be present in callus and stage II shoots without visible symptoms or medium turbidity	Norman and Alvarez (1994)

^a References summarized in Geier (1990) are not included.

Nitsch (1969) or MS (Murashige and Skoog 1962) basal salts, sugars, other organic components, and growth regulators were described for proliferation and plant regeneration from a variety of tissues. Axillary bud culture (Kunisaki 1980) proved to be another effective micropropagation method. Effects of explant and genotype on regeneration and genetic stability of regenerated plants have also been studied.

Recent studies describe callus, somatic embryogenesis, and protoplast culture. Caulogenic callus was induced from leaf and petiole segments of *A. scherzerianum*, multiple shoots were obtained, and rooting was induced (Liu and Xu 1992). *A. andraeanum* Hort. callus and shoot regeneration was reported by Lightbourn and Devi Prasad (1990), Kuehnle and Sugii (1991), and Singh (1991). Trends in these studies were similar to those reviewed in Geier (1990), where callus is induced on a modified Nitsch (1969) or Pierik et al. (1974) medium supplemented with only 2,4-dichlorophenoxyacetic acid (2,4-D) or a combination of 2,4-D and BA. Plant regeneration was achieved on medium with BA or devoid of growth regulators. A method for somatic embryo induction and conversion to plants was developed for *A. andraeanum* hybrids using in vitro lamina as explant sources (Kuehnle et al. 1992a). Preliminary work on anthurium protoplast isolation and culture was presented by Kuehnle and Nan (1991).

3 Micropagation

3.1 Establishment of Axenic Cultures

Disinfestation of anthurium tissues can be problematic, with the exception of seeds. Contamination rates usually range from 10 to 20% among leaf explants, 75% in spadix sections (Geier 1990), and 33 to 87% for excised axillary buds (Kunisaki 1980). Due to the protective layers of berry flesh and seed coat, disinfectant may be used at higher concentrations or longer exposure times with fruit and seeds. Slow-growing contaminants are not unusual in anthurium tissues and may appear after the first month of culture. Successfully disinfested explants should show minimal discoloration.

3.1.1 Seed

Seeds may be disinfested by first soaking the harvested berry in 3% sodium hypochlorite (NaOCl; e.g., 57.7% Clorox) for 15 min, followed by soaking excised seeds in 1% NaOCl for 20 min (Pierik et al. 1974). Each soak is accompanied by a 30-min rinse with several volumes of sterile water. The seed coat is then removed, and the explant, consisting of the embryo and endosperm, is cultured on appropriate medium (Pierik et al. 1974). Calcium hypochlorite can be substituted for sodium hypochlorite (Rosario and Lapitan

1981). Successful disinfestation has also been achieved by one soak of excised seed in 2.6% NaOCl for 5 min (Zens and Zimmer 1988) or LD disinfectant (Alcide Corp., Norwalk, Connecticut) at 1 part activator: 1 part base: 10 parts water for 30 min (Tanabe et al. 1989). Presence of a gelatinous or sticky substance often hinders the handling of the anthurium seeds with standard tissue culture tools. Although it is not usually present in seeds disinfested with NaOCl, removal of this substance is possible with a 13% sodium carbamate solution (Maurer and Brandes 1979). Seeds or excised embryos germinate within 4 weeks, with proliferation of callus usually occurring within 12 to 16 weeks (Rosario and Lapitan 1981).

3.1.2 Leaf, Spathe, and Spadix

Lamina, petiole, and spathe sections are generally disinfested by an initial dip in 70 to 95% alcohol, followed by a 10- to 30-min soak in 1.5 to 3% NaOCl. An alternative method uses a 5-min soak in 0.1% mercuric chloride solution with 0.25 ml/l Tween 20 in place of NaOCl (Eapen and Rao 1985). Similar methods are used for spadix explants, in which first the spathe surrounding the young spadix is disinfested, followed by disinfestation of the spadix proper (Geier 1982).

Unprotected or screenhouse cultivation of plants in subtropical and tropical areas is conducive to high contamination rates for field-grown material. In Jamaica, use of a 70% alcohol dip for 45 s and a 1.25% NaOCl soak for 15 min resulted in up to 70% contamination of leaf explants. Contamination was reduced to 10% by a presterilization soak in the fungicide Benlate (Dupont) (Lightbourn and Devi Prasad 1990). Axenic leaf blade and petiole cultures have been obtained using an initial 10-min soak in 0.14% Physan 20 (Maril Products Inc., Tustin, California) followed by consecutive soaks of 30 min in 0.53%, then 0.27%, NaOCl with one drop Tween 20/100 ml (Kuehnle and Sugii 1991); contamination rates of 5% are routinely achieved (T. Matsumoto, unpubl.).

While leaf callus produced from plants infected by *Xanthomonas campestris* pv. *dieffenbachiae* was found to be axenic, it should be noted that this bacterial pathogen can be harbored asymptotically in inoculated callus and shoots for 4 months to 1 year (Norman and Alvarez 1994).

3.1.3 Axillary Buds

Initial reports record a contamination rate of 33% using two soaks for 20 and 45 min in 0.53 and 0.27% NaOCl solution with Tween 20, respectively, with removal of bud scales (Kunisaki 1980). Reduction of exposure time to the disinfestant is possible through use of LD and Exspor disinfectants supplemented with 35% isopropyl alcohol (Alcide Corp., Norwalk, Connecticut; Tanabe and Matsumoto 1992).

3.2 Methods for Culture Initiation and Plant Multiplication

Enhanced axillary branching from microcuttings of in vitro shoots is used to multiply plants to the quantities desired. The initial shoots are obtained from a variety of sources, namely callus cultures, axillary buds, and somatic embryos. Several factors affect the success of anthurium micropagation, and some aberrations have been described among regenerated plants.

3.2.1 Callus

Callusing is used to initiate in vitro cultures of field material from which shoots for subsequent multiplication are derived (Fig. 2). This method is commonly used in The Netherlands and numerous reports have been published. Organogenic callus and plant regeneration have been achieved using seeds, embryos, and explant material of leaf lamina, petiole, spadix, spathe, and etiolated shoots (Geier 1990; Lightbourn and Devi Prasad 1990; Kuehnle and Sugii 1991; Liu and Xu 1992). In general, callus induction and proliferation are favored under dark conditions by addition of an auxin, usually 2,4-D, and a cytokinin, usually BA, to solid or liquid medium. Shoot proliferation from callus is stimulated with the removal of auxin from the medium, reduction of ammoniacal nitrogen, and increased light. Cytokinins such as 2-isopentenyladenine (2iP), BA, or kinetin may, in some cases, be required for shoot formation.

The soft tissue of newly unfolded leaves is successfully used for laminar explants. Explants of fully expanded leaves should include a major vein with

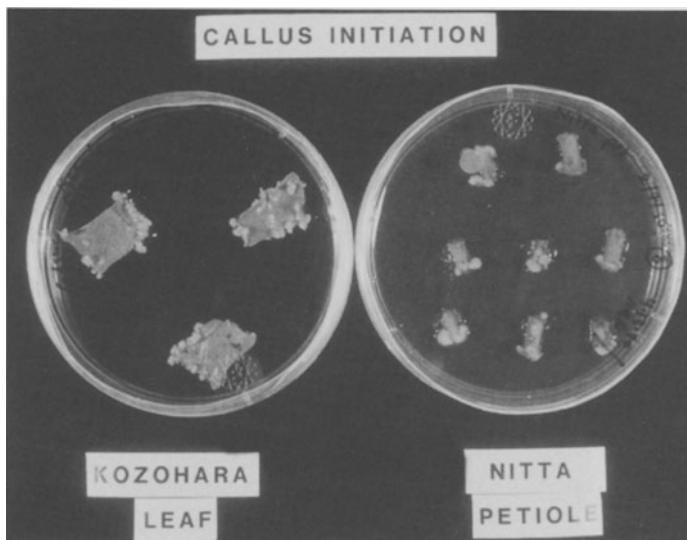


Fig. 2. Callus initiated from Kozohara lamina and Nitta petiole sections on modified Pierik medium with $0.36\ \mu\text{M}$ 2,4-D and $4.4\ \mu\text{M}$ BA with 0.18% Gelfrite in the dark

vascular tissue for improved proliferation (Finnie and van Staden 1986). It is suggested that young, unlignified leaves about one-half to two-thirds of the final length are most useful for *A. scherzerianum* (Geier 1990). Optimum regeneration in *A. andraeanum* occurs if leaves are harvested 1 day after they are fully expanded. Lamina sections show signs of proliferation as early as 2 to 4 weeks (Finnie and van Staden 1986) up to 12 to 16 weeks (Lightbourn and Devi Prasad 1990).

3.2.2 Axillary Bud

Direct shoot formation from excised buds was reported by Kunisaki (1980). This method establishes initial shoot cultures without an intervening callus phase, and thus may reduce the possibility of somaclonal variation and abnormal plant recovery, but at the expense of rapid propagation. According to Kunisaki (1980) and later adaptations (J. Kunisaki, pers. comm.) five to ten lateral buds are obtained from the stem of an anthurium plant (Fig. 3A), surface sterilized, and trimmed to 2 mm at the base. Shoot formation is encour-

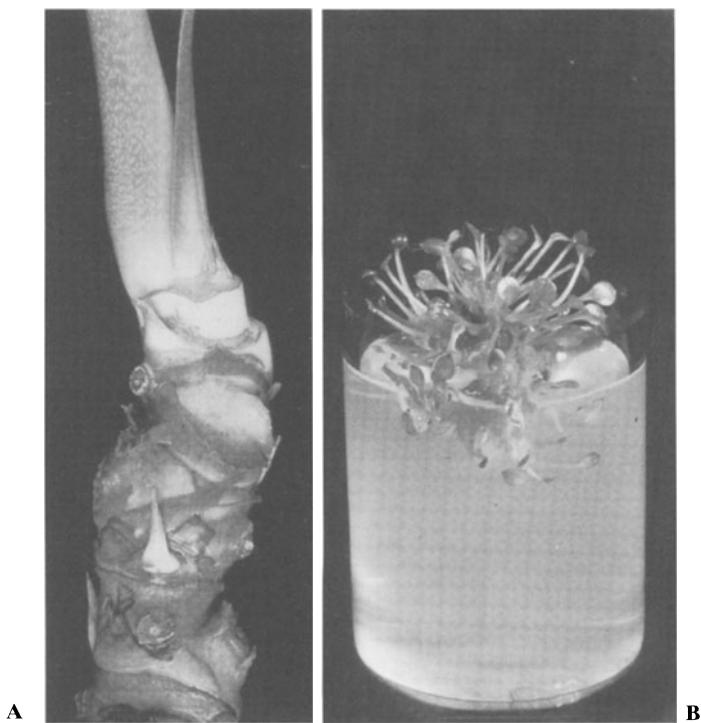


Fig. 3A,B. Axillary bud culture. **A** Stem section of mother plant, stripped of leaf sheaths to expose lateral buds. **B** Multiple shoot formation from stem sections of plants grown from axillary buds on modified half-strength MS medium with 2% sucrose and 0.89 μM BA. (Photos courtesy J. Kunisaki)

aged in a liquid modified MS medium consisting of MS salts at 3/8 strength, 15% coconut water, and 2% sucrose. After a single elongated shoot develops from each bud, usually within 12 to 18 months, a top cutting consisting of the apex and two or more leaves is cultured on a filter paper bridge in similar medium. For multiple shoot formation, basal portions of the remaining stem are placed on solid medium, or on a shaker in liquid medium, supplemented with 0.2 mg/l BA for a maximum of 2 months (Fig. 3B). Top cuttings taken from the multiple shoots are placed on medium used for initial shoot formation and solidified with 0.18% Gelrite for shoot growth and root formation. The basal explants are subcultured again to solid medium lacking BA for additional shoot formation. Once shoots form, top cuttings are taken once again and the remaining bases discarded.

3.2.3 Somatic Embryogenesis

Somatic embryogenesis and subsequent plant regeneration have been achieved in *A. andraeanum* hybrids. Whole lamina explants were harvested from in vitro-grown plants and plated on a modified half-strength MS medium with 2% sucrose, 1% glucose supplemented with 1 to 4 mg/l 2,4-D, and 0.33 to 1 mg/l kinetin. Induction of embryos (Fig. 4) and proliferation of secondary embryos occurred under darkness. Conversion and maturation occurred on the same basal medium plus 2% sucrose, 0.2 mg/l BA, and 0.18% Gelrite under a 16-h photoperiod (Kuehnle et al. 1992a). These plantlets can serve as a source of nodal microcuttings for subsequent multiplication.

In other reports, somatic embryogenesis in spadix callus of *Anthurium scherzerianum* was induced by lowering ammonium nitrate to 1.25 mM

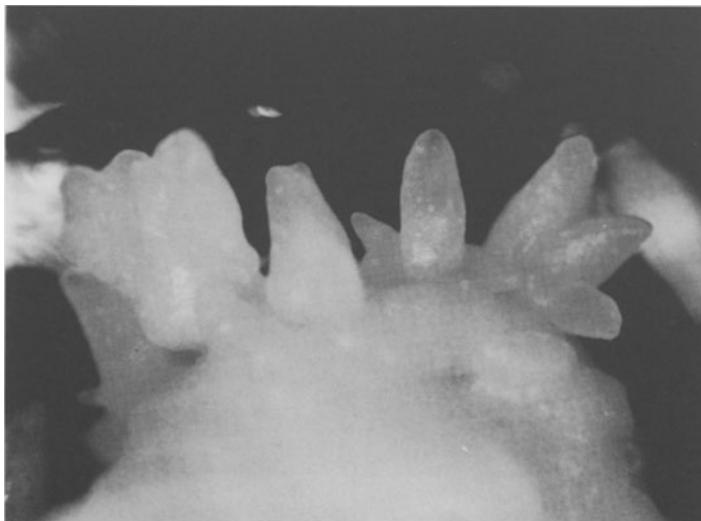


Fig. 4. Somatic embryos of Nitta cultured in the dark on modified half-strength MS medium with 2% sucrose, 1% glucose, 18 μ M 2,4-D, and 2.3 μ M kinetin

NH_4NO_3 in a Nitsch medium with $4.44\text{ }\mu\text{M}$ BA and $0.45\text{ }\mu\text{M}$ 2,4-D (Geier 1982). Somatic embryos germinated into bipolar structures and multiple plantlet clumps. However, these somatic embryos occurred sporadically (Geier 1990). Petioles from in vitro-grown plants of Lady Jane (*A. andraeanum* \times *A. antioquiense*) plated on a modified MS medium with $2\text{ }\mu\text{M}$ 6-benzylaminopurine (BAP), $2\text{ }\mu\text{M}$ zeatin, and $1\text{ }\mu\text{M}$ 2iP, also appeared to produce somatic embryos (F.J. Novak, pers. comm.). Cultures were kept in the dark for 2 to 3 months at 28°C and maintained on the same medium, either solid or liquid. Shoots were regenerated on a medium containing $0.5\text{ }\mu\text{M}$ IBA (indolebutyric acid).

3.2.4 Influence of Genotype and Selection of Explant

Genotype plays an important role in the multiplication and regeneration of anthuriums (Pierik et al. 1974). As with other crops, examples of variation among genotypes exist for anthurium callus formation (Geier 1990; Lightbourn and Devi Prasad 1990; Kuehnle and Sugii 1991; Liu and Xu 1992) and somatic embryogenesis (Kuehnle et al. 1992a). Differences in cultivar responses also apply to enhanced axillary branching, with the period of first leaf emergence ranging from 13 weeks for Hawaiian Butterfly to 38 weeks for Fuji Pink (Tanabe et al. 1991).

Selection of the explant is often dependent upon the material available and the objective of study. Limited number of axillary buds are available per individual mother plant, and recovery of the mother plant following removal of a large stem section for excision of axillary buds may take over one year. In contrast, greater amounts of propagative material are generally available for other methods, and removal of a leaf, spathe, or spadix will not cause substantial injury to a mother plant.

For clonal propagation, the use of seeds is highly discouraged due to the high variability in offspring from cross-pollinations. However, seed culture has been proposed as a breeding aid applicable to limited number of crosses or those with few resulting seeds (Tanabe et al. 1989). Clones are produced in vitro by promotion of multiple shoot formation from germinating seeds. Clones of each seed are evaluated by the breeder in the field; corresponding clones are retained in vitro. If and when the selection of a promising cultivar is made, propagules are already available in vitro for subsequent multiplication.

3.2.5 Rooting

Shoots will spontaneously root in culture under light conditions following removal or depletion of growth regulators, notably cytokinins, in the nutrient medium. If rooting does not readily occur, medium salts may be reduced to half the original strength and the solidifying agent may be reduced or eliminated. Some propagators include charcoal as a darkening agent in the rooting medium (N. van der Knaap, pers. comm.).

3.3 Acclimatization

Anthurium microcuttings rooted in vitro (termed stage III) prior to transfer to the greenhouse are generally hardier than unrooted propagules (stage II), but may be less cost-effective for the micropropagator or grower. Retail price per stage II microcutting is currently 20% less than stage III material for major labs in the USA. Success of acclimatization at stage II is often dependent on the particular cultivar. Many labs also sell acclimatized plants (Stage IV). These often ensure a better unit price per plant for the laboratory and increase survivability of plants for the buyer. Plants at any of these stages are susceptible to bacterial blight, rain and slug damage, and salt burn from use of undiluted complete fertilizer.

Stage II and stage III microcuttings should be carefully removed from the culture vessels and all remaining agar rinsed off. Stage II shoots preferably should contain the apex plus two or three leaves prior to transplantation ex vitro. Microcuttings are placed into a sterile, premoistened medium such as Oasis Root Cube, rockwool, or vermiculite/perlite combinations. Plants are kept in high humidity under 80% shade or 1500 to 2000 foot candles for approximately 2 months (Tanabe 1991). Stage III plantlets may be treated with a fungicide as a preplant soak or postplant drench (Imamura and Higaki 1981). Plants with intact roots are transplanted into a variety of media including Oasis or rockwool plugs, shredded tree fern fiber, or mixes of soil amendments such as composted shredded bark: no. 2 perlite (3:1 mix) under 80% shade with high humidity.

In large-scale commercial productions, misting or fogging systems are often employed to provide high humidity conditions conducive for further plant growth. Other methods include use of humidity tents for multiple flats (Fig. 5A) or clear plastic domes for individual flats (Fig. 5B). Plants are monitored and watered to prevent desiccation. The plastic cover should be removed in incremental stages, to gradually reduce the humidity (Tanabe 1991). Plants should continue to be protected from rain until large enough for transplant into beds or pots (Higaki et al. 1994).

3.4 Somaclonal Variation

Aberrations are occasionally observed among micropropagated plants. Although ploidy variation was found in callus of *A. scherzerianum*, carry-over to the regenerated plant was rare, based on stoma length, chromosome counts, and cytophotometry (Geier 1988, 1990).

Another source of variation may relate to the proliferation method used. Somaclonal variation was documented for plants obtained both from year-old callus pieces and from plants initiated by axillary bud culture and micropropagated long-term by enhanced axillary branching (Kuehnle and Sugii 1992; Kuehnle and Kuanprasert, unpubl.). Using 3 diverse genotypes, approximately 520 plants micropropagated via long-term callus culture (12 to 13 months) and 120 plants micropropagated via nodal cuttings, over a 3- to 4-year period, were grown to maturity and evaluated. Plants were grouped into

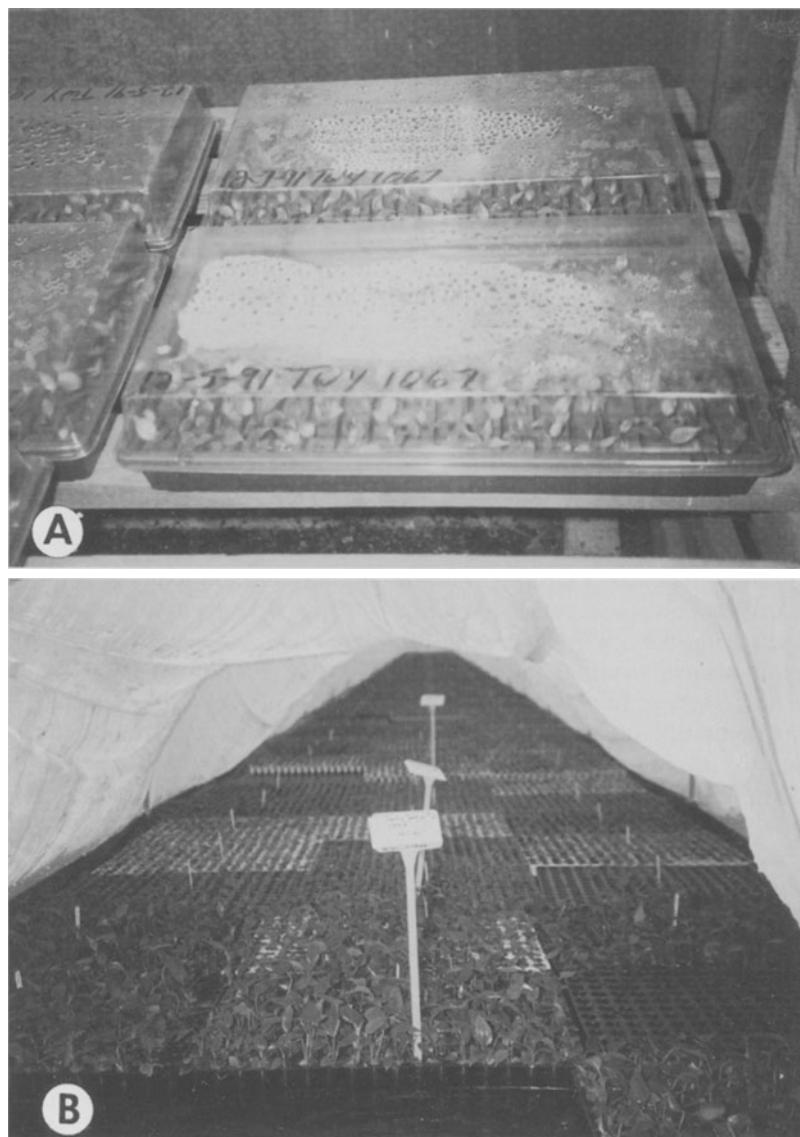


Fig. 5A,B. Acclimatization: **A** Stage III anthuriums transplanted ex vitro into trays containing Oasis rooting cubes and covered with plastic hoods. **B** Humidity tent used to cover mass plantings of anthurium

those arising from the same callus piece immediately prior to shoot regeneration, or those arising from the same nodal cuttings in the last subcultures prior to planting in the greenhouse.

Both sets of plants experienced stunting or shortening of internodes for all three genotypes. Shortened internodes occurred among 19 to 30% of the callus groups, but infrequently within a group, so that as few as 4% of the total

plants from a genotype may have had reduced stature. Plants from nodal cuttings showed a higher rate of stunting (25 to 43% of related groups), but no other aberrations. Among callus-derived plants, leaf shape abnormalities, leaf variegation or ploidy changes were detected in 0.8 to 2% of the total plants, corresponding to 3 to 8% of the original callus clumps put on plant regeneration medium; frequency varied among genotypes.

Based on these findings, it is suggested that callus proliferation be restricted to a few months prior to plant regeneration and that new stock plants for enhanced axillary branching, derived from callus or from axillary buds, be re-initiated at regular intervals of less than 3 years; every 6 to 12 months is practiced in The Netherlands.

3.5 Commercial Aspects (Table 2)

Among commercial tissue culture laboratories in 14 Western European countries surveyed in 1988, *Anthurium* ranked 14th among the most frequently propagated genera, with three countries producing more than 100 000 anthurium plants each (Pierik 1991). The Netherlands, which accounts for 29% of the West European production, micropropagated over 0.5 million plants of *A. andraeanum* in 1988, placing *A. andraeanum* second after *Gerbera* in quantity of plants produced for the cut flower industry. For the potted plant market, *A. scherzerianum* ranked fourth, after *Nephrolepis*, *Saintpaulia*, and *Ficus*, with 1.7 million plants produced in vitro in The Netherlands (Pierik 1991). Figures for the quantity of plants micropropagated from 1988 to 1990 have been compiled and are shown in Table 2 (R.L.M. Pierik, pers. comm.). In The Netherlands, initial multiplication relies largely upon callus culture.

To estimate the extent of *Anthurium* micropropagation in the USA, a survey was sent to major commercial tissue culture laboratories in Hawaii and the continental USA. Of seven laboratories currently producing over 10 000 anthuriums per year, an estimated 3.8 million plants were micropropagated in 1993, with three of these labs producing 90% of the supply with over 1 million plants each. The majority of plants were for use in cut flower and flowering potted plant production; fewer were produced for the foliage industry. A variety of *A. andraeanum* Hort., *A. scherzerianum*, and hybrids with *A. amnicola* and *A. antioquiense* are produced for the flowering potted plant market. All seven laboratories rely primarily on axillary and apical bud culture

Table 2. Quantity of micropropagated anthurium plants in The Netherlands, 1988 to 1990. (Courtesy R.L.M. Pierik, Agricultural University, Wageningen, The Netherlands)

	1988	1989	1990
<i>A. andraeanum</i>	509 300	919 000	1 516 500
<i>A. scherzerianum</i>	1 684 624	2 454 400	4 157 750
Total plants	2 193 924	3 373 400	5 674 250

for culture establishment, followed by axillary branching for multiplication; two of seven (29%) labs use callus or somatic embryogenesis as a supplemental form of culture establishment. The vast majority of plants sold are derived from initial bud culture. Microppropagated anthurium plants are sold by 71% of the laboratories as stage III or stage IV; 29% of the labs also offer stage II microcuttings. In terms of total plants sold, stages III and IV are slightly more popular, with an estimated 35% stage III, 37% stage IV, and 28% stage II sold by the seven laboratories.

4 Summary and Conclusions

Methods for fast clonal propagation are essential to fulfill growers' demands for anthurium plants worldwide. Heterogeneous seed progeny and slow methods of field propagation are unsuitable for generating the large quantities needed. Efficiency of callus culture has been greatly improved for many genotypes since this system was first described for anthurium in 1974 by Pierik et al. Alternative methods, such as axillary bud culture and somatic embryogenesis, have also been developed. Currently, all three methods are commercially used for microp propagation of cut flower and potted plant anthuriums. Slow response of certain genotypes is still a problem despite modifications to the culture medium. Mass propagation linked with disease-indexing programs are currently being investigated, and will be beneficial to production areas plagued with the bacterial blight. In the future, in vitro regeneration of transgenic plants might play an important role in breeding for disease resistance and other desirable qualitative traits.

5 Protocol

5.1 Axillary Bud

1. Excise buds with a 1-cm base from a stem which has been washed with soap, soaked in 0.53% NaOCl for 5 min, and air-dried for 2–3 days.
2. Soak in 0.53% NaOCl for 30 min, rinse, then remove two to three leaf sheaths and cut to 2 mm.
3. Soak again in disinfestation solution for 5 min and rinse in sterile water.
4. Place bud in liquid half-strength MS medium with 2% sucrose, 15% coconut water in culture tube on rotary drum at 0.2 rpm, under light, until shoot elongates with leaves.
5. Use basal stem cutting for enhanced axillary branching on half-strength MS medium with 2% sucrose and 0.2 mg/l BA under light; shoots are rooted on hormone-free medium in light.

5.2 Callus

1. Surface sterilize petiole and lamina sections in 0.14% Physan 20 for 10 min, 0.53% NaOCl for 30 min, and 0.26% NaOCl for 30 min, then rinse in sterile water.
2. Plate on solid modified MS medium containing 1 mg/l BA and 0.08–0.1 mg/l 2,4-D in dark.

3. After several months, explants with callus are moved into light for shoot proliferation on BA medium.
4. Cuttings are taken for enhanced axillary branching and rooting, as in 5.1.5, above.

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I.3 Micropropagation of *Blandfordia* Species (Christmas Bells)

K.A. JOHNSON and M. BURCHETT¹

1 General Account

The genus *Blandfordia* (family Blandfordiaceae) comprises four species; *B. cunninghamii* Lindl., *B. grandiflora* R. Br., *B. nobilis* Sm. and *B. punicea* (Labill.) Sweet. It is endemic to eastern subtropical and temperate Australia (Elliot and Jones 1982; Knees 1986; Henderson 1987). This genus is fast gaining importance in the horticultural industry as a cut flower crop of great beauty (Moody 1993), and has a potential to become a new pot plant variety (Wrigley and Fagg 1988; Johnson 1993). The genus is already being exploited in a small way for the domestic and overseas cut flower markets (Stackhouse 1987; Moody 1993); however, none of the species is widely cultivated. *B. grandiflora*, the most striking, is being picked from its natural habitat, particularly for the pre-Christmas markets, under a licensing system operated by the Australian National Parks and Wildlife Services. However, with the growing popularity of the flower, its diminishing natural populations, as a result of destruction of habitat and its erratic postfire behaviour (Johnson et al. 1994), the long-term survival of the species is in doubt.

As a genus, *Blandfordia*, it appears has a chance of survival only in areas which are especially set aside as "Blandfordia country", and as a cultivated horticultural crop. Conducted field surveys indicate very clearly that much of the natural *Blandfordia* habitat has been eliminated by land use incompatible with the survival of natural populations. Most populations of the genus on private land have already disappeared. Although they are permanently protected in national parks, their future in state forests and council reserves is very uncertain, depending on future decisions on land use in those areas.

The first two species of *Blandfordia* were described and named at the beginning of the 19th century. The originator of the generic name, Sir James Edward Smith, named *Blandfordia* in honour of George, Marquis of Blandford (Ingram 1965). *Blandfordia nobilis* Sm., the least showy one of all, was collected in 1800, and entered cultivation in 1803 as a glasshouse plant in England (Cavanah 1993). In 1810 the first *B. grandiflora* R. Br., also known as *Blandfordia flammea* (Lindley ex Paxton 1849) (Ingram 1965), was collected

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by Hunter near Newcastle, New South Wales (NSW), and described by Brown (1810) in *Prodromus Florae Novae Hollandiae et insulae Van-Diemen*.

The third species to be described, *B. punicea* (Labill.) Sweet, was originally described as *Aletris punicea* by Labillardiere. It was transferred to *Blandfordia* some 25 years later (Brown 1810). *B. cunninghamii* Lindley was named after Allan Cunningham (1791–1839), botanist and explorer, who made extensive collections in Australia and New Zealand.

Blandfordia is a genus of perennial herbs with basal leaves and a single terminal racemose inflorescence. The leaves are linear and mostly crowded at the base of the stem with crenulate or smooth margins. The flowers are pediculate, pendulous, each subtended with one bract and a smaller bracteole. They are bisexual, and tubular to campanulate in shape with the colour ranging from red to yellow (see Fig. 1) (Harris 1949, 1986; Harrison 1953; Beadle et al. 1986; Henderson 1987). All species flower from early to mid-summer, but flowering is known to be dependent on fire occurrence (Johnson et al. 1994). The genus is very distinctive, with the rhizomatous corm and long contractile roots, consistent shoot morphology and showy bells, and whereas it was previously included in family Liliaceae, it is now recognised as a monogeneric family by Dahlgren et al. (1985), Brummit (1992) and Quirico (1993).

B. grandiflora R. Br., *B. nobilis* Sm., *B. cunninghamii* Lind. and *B. punicea* (Labill.) Sweet are endemic to eastern Australia. They are distributed along the eastern coast of Queensland, NSW and Tasmania, with *B. grandiflora* occurring on the mainland and Fraser Island from 24°S to 34°S and *B. nobilis*



Fig. 1. *Blandfordia grandiflora* vary in flower attributes, such as colour, shape and number of bells amongst others, which are important in selection for cut flower production. This photograph illustrates one of many of these different combinations of flower attributes

occurring from 34°S (Sydney, NSW) to approximately 36°S. *B. cunninghamii* occurs in the Illawara and the Blue Mountains regions of NSW (34°S) and *B. punicea* is endemic to Tasmania.

1. *Blandfordia cunninghamii* Lind (named after Cunningham) grows at higher altitudes ranging from 500 to 960m. It is a very uniform species; the flowers are borne on erect stems up to 1m long with bells up to 60mm in length. The colour of the bells is usually red with yellow wings on internal lobes. Plants usually grow in small clumps less than 0.5m in diameter, and flower in summer. The leaves of this species are much wider in comparison to other species occurring in NSW and Queensland; they are much longer, wider, softer to touch, and they are very flexible. In many ways they are similar to *B. punicea* leaves. *B. cunninghamii* grows in moderately wet forest with rainfall of 800–1000mm, and temperature range from –3 to 38°C. This species has been listed by Briggs and Leigh in 1988 as a rare and threatened Australian plant. Habitats of *B. cunninghamii* have damp soil (Beadle et al. 1986; Quirico 1993) and include hanging swamps (Baker et al. 1984), rock ledges and in heath (Farley and Moore 1989).

There could possibly be hybrids occurring between *B. grandiflora* and *B. cunninghamii* in their overlapping ranges in the Blue Mts. regions, NSW (Higher and Lower Blue Mts.). These were recorded as *B. grandiflora* var. *elongata* Benth. in 1878 (Henderson 1987).

2. *Blandfordia grandiflora* R. Br. is a variable species in terms of colour. Its flowers are yellow to orange or red, and of various combinations of these colours. The stem is up to about 1m long with leaves to about 70cm and the bells 35–80mm long (Beadle et al. 1986; Quirico 1993). Its natural distribution ranges from Gosford (NSW) to Fraser Island in Queensland. Its natural habitats are the moist areas in coastal heathland of annual rainfall 1000–1600mm, and a temperature range from 0–35°C. *B. grandiflora* can also be found at Gibraltar Range (NSW) at altitudes above 800m. The yellow flowers are dominant in Queensland populations, hence the name *Blandfordia flammea* (Lindley ex Paxton) or *B. aurea* Hook in previous classifications (Henderson 1987).

3. *Blandfordia nobilis* Sm. grows in the Narrabeen and Hawkesbury sandstone country (NSW), in coastal heath and in the mountains, but not in the cold, dry regions. It is also found in open patches of pure and with good rainfall, in water seepage areas and swampy heath. The annual rainfall is approximately 1200mm per annum, and temperature ranges from average 12.5 to 22.0°C. The flowers are of many shades of red, and they can be up to 40mm long and about 1cm wide, arranged in a cluster of flowers at the top of the main stem. The stem is up to 60cm long, with leaves to about 75cm long (Beadle et al. 1986).

4. *Blandfordia punicea* (Labill.) Sweet. In Latin, *puniceus* means carmine-red or crimson, and indeed this species is very uniform with regard to colour, being scarlet on the outside and golden on the inner surface. The perianth inner lobes have brilliant yellow wings. Flowers are about 50mm long, and all are also of a very uniform shape, numerous in large terminal racemes on erect stems 30cm to 1m tall. The leaves are prominently veined, with serrulate,

recurved margins and of dark green colour. They are about as long as the inflorescence (Curtis 1963). *B. punicea* is endemic to Tasmania. It is found in the northwest, being restricted to the coastal area at Rocky Cape National Park. Its altitudinal distribution ranges from sea level to subalpine regions, occasionally reaching 1300m. The rainfall in these areas ranges from 1000–3000mm and the temperature varies from 10–38°C. Its natural habitats are sandy and acid heath at sea level, moors, swamps and hillsides, where it is found growing in cracks in quartzite. *B. punicea* has also been found in the most adverse conditions on Maatsuyker Island, which is located about 10km off the Tasmanian south coast at latitude 43°40', longitude 146°22'.

2 Conventional Propagation and Need for Micropagation

Although *Blandfordia* has been popular for years as a cut flower, propagation by means other than from seed has not been widely investigated. Seed-propagated plants, however, create a wide range of characteristics quite often not desired in the cut flower production. *Blandfordia* can also be propagated vegetatively from the underground rhizomatous corm with highly impressed internodes. The central growing point is located at the centre of the crown, and becomes active when flowering occurs. At the sides and around the crown of



Fig. 2. *Blandfordia* rhizomatous corms (with contractile roots) can be used for vegetative propagation, but the process is time-consuming. Arrow indicates a new corm which could be separated for vegetative reproduction

the corm, the side buds may arise. These may produce young shoots which give rise to a new clone, but this method of propagation is very slow. Clumps of *Blandfordia* can be subdivided into single plants every 2–3 years giving two or three new plants, which take about 2 years to regain the flower production stage (BREG, pers. comm.; Fig. 2).

The great variation in seedling material in terms of floral attributes can be avoided by using vegetative propagation by splits, but this process is impractical because of the time involved. Micropropagation has become a propagation tool to build up quickly the stock selected clones of desired attributes. Also the need for the development of new hybrids, resulting in plants bred for qualities such as vigour, yield and consistency of performance, makes micropropagation a desired technology for the cultivation of the genus. From the commercial perspective, the need for supply of not only the quantity, but also the quality, of the propagation material is of prime importance at this early stage of *Blandfordia* crop development. The development of this genus, for both domestic and overseas markets, as cut flowers, pot plants or garden specimens, and its conservation, which will presumably involve both ex situ and in situ cultivation, both require speedier approaches than those of conventional propagation methods alone.

3 Micropagation

The initial tissue culture investigations with *Blandfordia* dealt with seed germination in vitro and also an attempt to initiate shoot tip culture in vitro. Dunstan (1982) germinated *B. nobilis* seeds in vitro, but was not able to obtain clean cultures of dissected shoot apices. The importance of and the need for a new propagation technology of *Blandfordia* has been recognised since 1987, and tissue culture technology has become a tool for a commercial propagation of this genus (Johnson 1987, 1990a,b,c, 1992, 1993, 1994; Johnson and Burchett 1991).

Our investigations aimed to develop micropropagation procedures of *Blandfordia* species, the long-term aim being to relieve the pressure on wild populations and increase the availability of plant material. Such material is required for future selection, propagation and breeding programmes. The appropriate media for multiplication and rooting were selected from a very wide range of media used in micropropagation experiments. *B. grandiflora*, the species with greatest immediate potential to become a new crop for the international cut flower markets, has been used as a model in these experiments (Johnson and Burchett 1991).

3.1 Sterilization

A three-step sterilization procedure was applied. Actively growing shoot tips were removed from selected clones after 6 weeks of plant stock exposure to an

environment drier than the glasshouse (Fig. 3). The shoot tips were individually washed with soap and water and the sterilization procedure followed. In the first step, 1% (v/v) chlorine solution [4% (w/v) calcium hypochlorite] was applied for 20 min and the first set of leaves was subsequently removed. In the second step, plant tissue was exposed to 0.5% (v/v) chlorine solution for 10 min and then the second set of leaves was removed. In the third step, 0.05% (v/v) chlorine solution was used for 5 min with the removal of the third set of leaves. Shoot tips were rinsed three times with sterile water after the first and second step of sterilization procedures. Dissected tips of about 5 mm in length were given a final quick rinse in 0.05% chlorine solution before they were placed in the tube vessels.

3.2 Media and Incubation

Suitable media for the culture of bud explants are summarised in Table 1. Fifty-ml tube vessels and 200-ml jars containing 10 and 50 ml of the selected medium respectively were used. After adjusting the pH to 5.7 with 0.1 M NaOH, all media used in these experiments were sterilized at 120°C for 15 min.

Explants were incubated at $26 \pm 2^\circ\text{C}$ during the day and $24 \pm 2^\circ\text{C}$ at night with a light intensity of $50 \mu\text{mol/m}^2/\text{s}$ for 16 h in the multiplying stage and $60 \mu\text{mol/m}^2/\text{s}$ at the rooting stage.

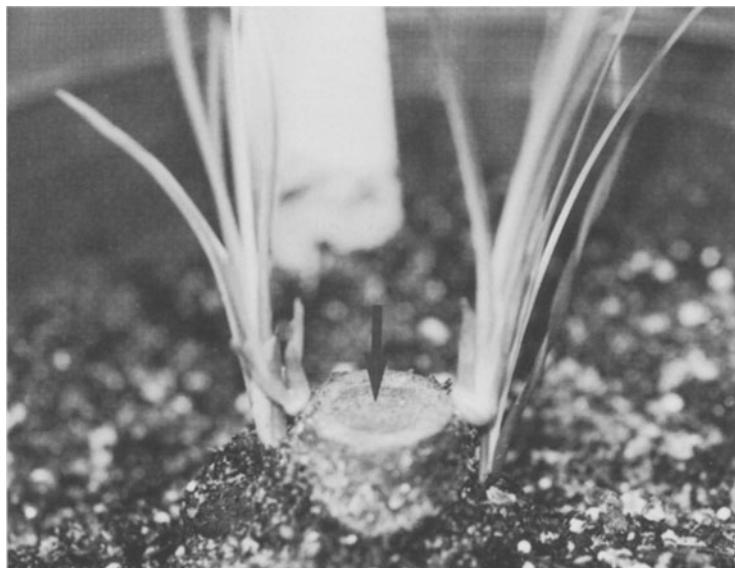


Fig. 3. *Blandfordia* stock plant after removal of the apical shoot tip. The removal of the leading shoot enhances the development of axillary shoots, which become the secondary source of explant material. Arrow indicates the area from which the apical shoot was removed

Table 1. Mean fresh weight (g), shoot number and shoot length (cm) of *B. grandiflora* after 18 weeks treated with kinetin, BAP and 2iP

Treatment	Cytokinin used	Concentration ($\mu\text{M/l}$)	Fresh weight (g)	Shoot number	Shoot length (cm)
1	Control	0	1.7978 a ^a	1.7931 a	4.1611 f
2	Kinetin	0.5	2.1539 ab	2.6872 b	3.4333 e
3	Kinetin	2.0	2.5850 b	3.6852 bc	2.544 d
4	Kinetin	8.0	4.3694 cd	7.8864 d	1.4222 ab
5	BAP	0.5	5.0267 de	7.4771 d	1.9222 bcd
6	BAP	1.0	4.3872 cd	9.7820 e	1.0000 a
7	BAP	2.0	3.8239 c	8.2728 de	1.0500 a
8	BAP	8.0	2.7522 b	4.2271 c	1.1944 ab
9	BAP	32.0	2.7422 b	3.8909 c	2.5500 d
10	2iP	2.0	3.7033 c	6.7229 d	2.3778 cd
11	2iP	8.0	4.7794 de	8.0753 de	2.1722 cd
12	2iP	32.0	7.5694 f	6.8748 d	2.3333 cd
13	2iP	128.0	5.4478 e	7.0386 d	1.6461 abc

^aMeans with the same letter are not significantly different from one another using Duncan's multiple range test; $P \leq 0.05$. Each mean represents six replications.

In preliminary studies it was observed that *Blandfordia* shoot tips can be maintained on the basic medium consisting of MS (Murashige and Skoog 1962) with macro-, and micronutrients, vitamins, *myo*-inositol, sucrose 3% (w/v) and Gelrite as a solidifying agent 0.2% (w/v). As the work progressed, we established the protocol, which supports good multiplication and rooting. The maintenance of shoot tips on basal MS medium for a period of 1 week at 27°C in a dark incubator is a good screening procedure for any exogenous contaminants.

3.3 Shoot Proliferation

The effects of 6-furfurylaminopurine (kinetin), 6-benzylaminopurine (BAP) and 6-isopentylaminopurine (2iP) on multiple shoot formation, average fresh weight (mass) and shoot elongation were investigated. Further investigations included an addition of the liquid phase medium on top of the solid phase without the removal of the developing plantlets. The objectives of this experiment were simply to find out whether an additional layer of liquid medium containing some cytokinins would increase shoot multiplication, hence the fresh weight, and at the same time whether it would save labour and time.

The results of the first experiment indicated that the best multiplication rates were achieved with kinetin and 2iP at a concentration of 8 μM , giving eight shootlets per explant, and with BAP at 2 μM , giving ten shootlets per explant. There was no further increase in multiplication when 32 and 128 μM of 2iP was applied but there was increase in the mean fresh weights (masses), which is indicative of the vegetative (elongation) growth (Johnson and Burchett 1991; Table 1).

Maene and Debergh (1985) reported that the addition of liquid medium to established tissue cultures improved elongation and rooting of selected plant cultivars. This method was applied to the *Blandfordia* multiplying material with the aim of increasing multiplication rates. The multiplication of *B. grandiflora* explants increased after 165 days in culture, without cutting, with an addition of the liquid phase medium consisting of half-strength MS and $0.5 \mu\text{M}$ BAP gave 27 shootlets per explants, and 26 shootlets per explants on half-strength MS with $1 \mu\text{M}$ BAP. Higher concentrations of BAP in liquid phase had the reverse effect. BAP at 8 and $16 \mu\text{M}$ became toxic to the clonal material and the original shootlets turned into a vitrified mass of tissue (Fig. 4). Differences between treatments are shown in Fig. 5. There was a significant difference in the multiplication of the *B. grandiflora* when liquid phase consisting of half-strength MS and kinetin at higher concentration was used. An increase from 3 ($0.5 \mu\text{M}$ kinetin) to 14 shootlets per explant on $16 \mu\text{M}$ of kinetin was achieved. The experiments described here showed very clearly that the addition of the liquid phase medium could be applied to improve the multiplication and elongation of *B. grandiflora* shootlets. The results also showed clearly that as little as $0.5 \mu\text{M}$ BAP can increase shoot multiplication and, in addition, the liquid phase can save the intensive labour usually required in traditional in vitro production systems (Johnson 1992). The semi-automated system described by Vanderschaeghe and Debergh (1987) could be easily applied to micropropagation of selected cultivars of *B. grandiflora*. The observed improvement in growth of cultures where transfer of plants is avoided by the addition of a supplementary liquid phase may well be due to avoidance of the transitory loss in continual growth and development. Vieitez et al. (1989) applied liquid phase to improve micropropagation and also to save labour, when working with *Camellia japonica* cv. Alba plena. This procedure was also applied to exotic monocots, *Hyacinthus orientalis* cv. Carnegie, Bach and Cecot (1988, 1989), for example, reporting beneficial effects of double-phase culture medium. Decreased handling costs and increased number of prolifer-

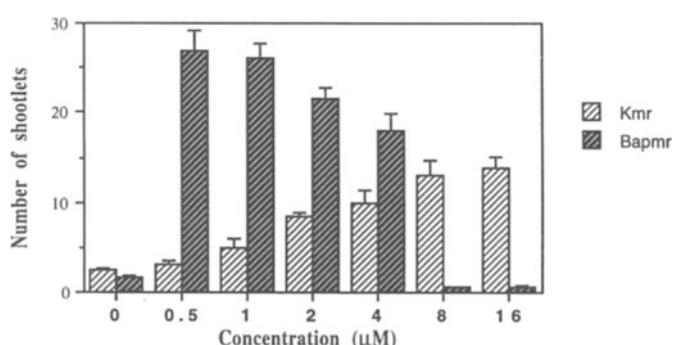


Fig. 4. Number of shoots grown on the medium with the additional layer of the liquid phase. Observe the difference in the number of shootlets on different concentrations of BAP (*Bapmr*) and kinetin (*Kmr*) respectively. The concentrations of growth regulators are expressed in $\mu\text{M/l}$. Vertical bars represent standard errors at $P \leq 0.05$

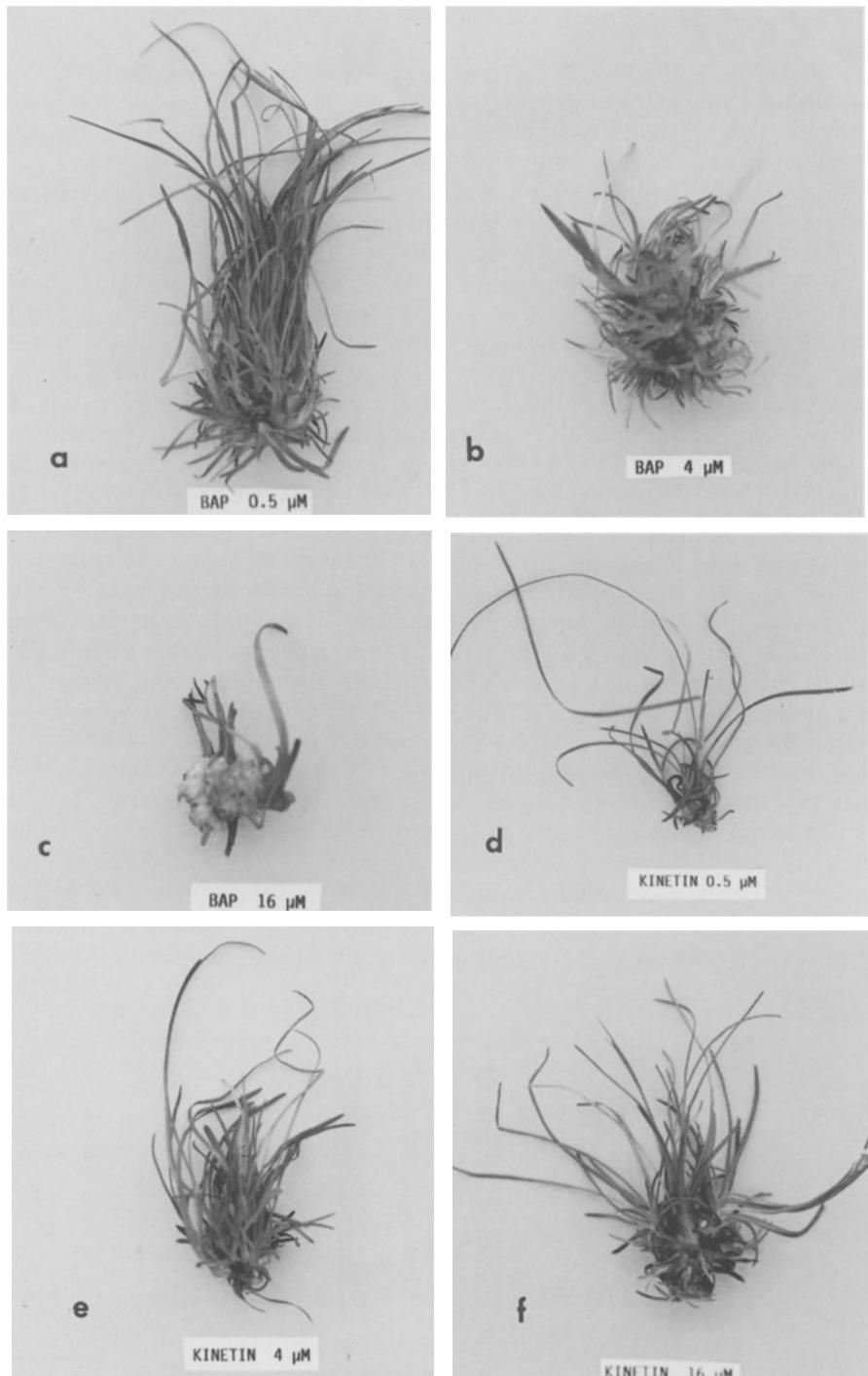


Fig. 5a-f. Shoot tip proliferation of *Blandfordia grandiflora* after 165 days in the liquid phase medium. (a) 0.5 μ M, (b) 4 μ M, (c) 16 μ M concentrations of BAP, and kinetin (d) 0.5 μ M, (e) 4 μ M and (f) 16 μ M, respectively. Observe retarded growth of the plant tissues on 16 μ M BAP (c)

ated buds makes this method an invaluable and a very economical tool for future large-scale production of geophytes.

3.4 Rooting, In Vivo Establishment and Storage

For root formation, indolebutyric acid (IBA), naphthaleneacetic acid (NAA) and indole acetic acid (IAA) were tried. Sixty seven percent of *B. grandiflora* microcuttings rooted on basic MS medium containing no auxin. When 2 and 8 μM of IBA or 2 μM of IAA were used, 100% of the microcuttings rooted with an average of six roots per microcutting (Fig. 6).

A pasteurised potting mix, consisting of 1 part sand:1 part peat:1 part perlite was used for the establishment of *B. grandiflora* plantlets. These plantlets were placed under mist with the natural light reduced to 70% with a shade cloth and sprayed with Previcur (Shering PTY LTD. Agrochemicals) to avoid fungal contamination. At the end of the first 4 weeks, a half-strength Aquasol liquid fertiliser was applied on a twice-weekly basis. The average survival rate was 90%. All plantlets with roots initiated in vitro survived transplanting to glasshouse conditions.

The initial long-term storage trials have shown that *Blandfordia* species maintained on a basal medium for 2 weeks under prescribed temperature and light conditions can be stored successfully at 10°C for 8 months under dark conditions.

3.5 Commercial Aspects

The formation of a group of growers, researchers, advisers and other interested parties, in 1989, known as Blandfordia Research and Extention Group

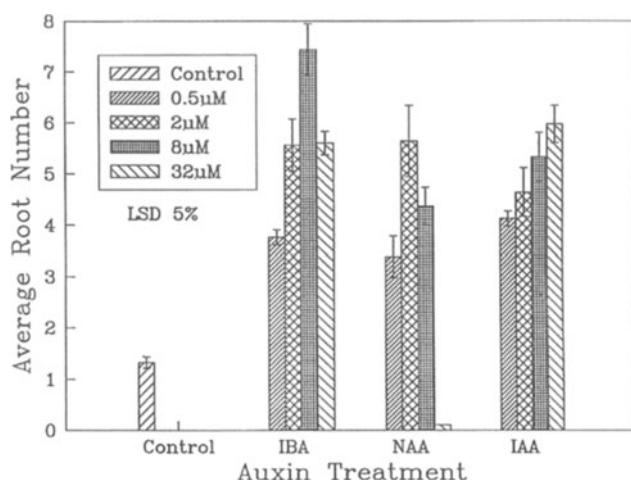


Fig. 6. In vitro responses of *B. grandiflora* shootlets to different concentrations of IBA, NAA and IAA. The concentrations are expressed in $\mu\text{M/l}$. Vertical bars represent standard errors at $P \leq 0.05$

(BREG), has consolidated its efforts to develop and market *Blandfordia* as a commercial cut flower. This is an innovative way of encouraging communication and cooperation, which aims to develop a new crop (Lamont and Maddock 1989). At present, *Blandfordia* is cultivated by a small number of growers in northern New South Wales, and some blooms are still harvested from the wild under licence. Flowers harvested from the wild are sold on the domestic market only.

Blandfordia flowers October to January (early summer in Australia, Christmas). A trial shipment of the *Blandfordia* flowers to Japan began in December 1991, and further shipments have been made each year since. The price at the Tokyo flower auction reached \$A 5 per stem in 1991, with an immediate order for 20000 stems per week for the Tokyo market alone. Exported blooms paid about \$1.50 per stem in 1994 and 1995. Only cultivated flowers are of export quality and can meet Japan's phytosanitary requirements. The flowers were also shipped to Holland for the first time in 1993 and were very well received (BREG, pers. comm. 1994). At present, three Biotechnology Companies are involved in micropropagation and trials of selected clones. These clones were released on contract bases from the University of Technology, Sydney. New selections are being made and are being micropropagated for future trials. *Blandfordia* is very slow-growing, however, and the commercialization is still very much in the trial-and-error stage. There is an exciting future for this crop.

4 Summary and Conclusions

So far, only shoot tip culture has been investigated for the regeneration of *Blandfordia* plants. Establishing the in vitro protocol for *B. grandiflora* has already had positive consequences. The very first selected clones of *B. grandiflora*, after being established in vitro, have been planted out in the nursery and are awaiting evaluation for vigour, and flowering. It can be reported that the very few *B. cunninghamii* seeds found in the Blue Mountains (NSW) have also been successfully germinated in vitro using the *B. grandiflora* multiplication procedure. However, the protocol used did not give results as in multiplication of *B. grandiflora*, consequently this first protocol for the genus *Blandfordia* may have to be modified to suit each individual species, hybrid or even clone, since the different behaviour of other established in vitro species and clones has already been observed. Finally, the first *Blandfordia* F₁ hybrid seeds of *B. grandiflora* × *B. grandiflora*, *B. grandiflora* × *B. nobilis*, *B. grandiflora* × *B. punicea*, which were obtained by hand pollination, have germinated in vitro, were successfully planted out, and will be evaluated for their floral attribute once they flower.

In 1989, Podger and Brown, studying damage caused by *Phytophthora cinnamomi* on disturbed sites in temperate rainforest in western Tasmania, found the *B. punicea* is very susceptible to *P. cinnamomi* attack. Another

important investigation conducted by Coombs and Ramsey (1991) suggested that this genus may act as host to an insect pest, *Helicoverpa armigera*, which damages the flower buds. These observations have proven correct in commercial crop-growing by the industry. Therefore efforts should go into developing techniques for resistance to these already known diseases and pests.

5 Protocol for Growing *Blandfordia* Plants In Vitro

Explant	Basal medium	Phase	Growth regulators (μM)	Growth response
Step 1 (initiation)				
Shoot tips	MS	Solid	O	None
Step 2 (multiplication)				
Shoot tips	MS	Solid	BAP 2	Shootlets
	MS	Solid	Kinetin 8	Shootlets
	MS	Solid	2iP 8	Shootlets
Step 3 (multiplication)	1/2MS	Liquid	BAP 0.5	Shootlets
Step 4 (rooting)				
Shootlets	MS	Solid	IBA 2-8	Roots
		Solid	IAA 2	Roots

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I.4 Micropropagation of Ornamental Bromeliads (Bromeliaceae)

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1 Introduction

The family Bromeliaceae contains about 2500 species native to tropical North and South America (Dimmitt 1992). The regions richest in bromeliads are Mexico, the Antilles, Costa Rica, eastern and southern Brazil, the Andes of Colombia, Peru, and Chile (Rauh 1990).

One of the most prominent characteristics of bromeliads is the presence of leaves arranged in a rosette. In most bromeliads the stem axis is short and compact (Rauh 1990). The foliage can present several varied color patterns, ranging from solid green to barred with maroon or gray cross-bands. The beautiful inflorescences also vary in form and color combinations (Dimmitt 1992).

The pineapple represents the best-known bromeliad; its culture has great economic importance for many tropical and subtropical countries. The pineapple was considered particularly in *High-Tech and Micropropagation II* (Moore et al. 1992). However, most bromeliads are known for their decorative leaves and flowers and have been used as ornamental houseplants for over a century (Padilha 1986).

Bromeliads are divided into three subfamilies, the Pitcairnioideae being considered the most primitive among them. This subfamily contains genera such as *Puya*, *Dyckia*, and *Pitcairnia*. Almost all species belonging to this group of plants are terrestrial or saxicolous and many of them are being increasingly used as ornamental plants. The subfamily Bromelioideae presents the greatest variety of bromeliad forms, ranging from terrestrial to epiphytic plants. These bromeliads possess a well-developed water reservoir at the base of the leaves (tank bromeliads), which seems to counteract in part the poorly developed root system.

The genera *Aechmea*, *Ananas*, *Billbergia*, *Cryptanthus*, *Neoregelia*, and *Nidularium* are the best known horticulturally, being used as both indoor and outdoor plants. Neoregelias, nidulariuns, and canistruns plants present in general beautiful and attractively colored leaf bracts surrounding their inflorescences during the period of flowering (The Bromeliad Society 1977). Furthermore, some bromeliads of this subfamily provide fibers that support a

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sizable commercial industry, such as *Neoglaziovia variegata*, endemic to Brazil (Benzing 1980). The subfamily Tillandsioideae includes a great number of epiphytic members, with genera such as *Guzmania*, *Tillandsia*, and *Vriesea* being valued highly in the ornamental bromeliad market. The so-called atmospheric tillandsias have no leaf water tanks. In these plants the moisture and salts can be absorbed from the air through specialized trichomes which cover the surface of the leaves, giving them a brilliant silver color. They are commonly used as ornamental bromeliads (The Bromeliad Society 1977). Most guzmania and vrieseas are native to the rain forest, and many species present highly decorative foliage, inflorescences, and forms. For this reason, they have been intensively collected from the wild for sale or private use. Furthermore, the native forests in which they grow have been progressively destroyed, thus increasing the risk of extinction of many bromeliads (Benzing 1980; Mercier and Kerbauy 1995).

In addition to their ornamental value, several bromeliad species possess medicinal properties as vermifuges (*Bromelia pinguin*), diuretics (*Pitcairnia pungens*), and anti-inflammatories (bromelains from pineapple and other bromeliads) (Benzing 1980). However, the bromeliads are, first of all, beautiful plants, the foliage, shape, and inflorescences of some of them make the Bromeliaceae a very remarkable family.

Micropropagation of bromeliads is in high demand because of the superior quality of the plantlets when compared to seedlings, programmable flowering, and uniformity. However, the total procedure in obtaining an acceptable product takes a long period before the plants can be delivered to the grower (Capellades Queralt et al. 1991). In Australia and New Zealand, bromeliads are currently available from micropropagation companies (Barlass 1991). In The Netherlands, the production of bromeliads as pot plants reached 289 300 plants in 1988. *Cryptanthus* is one of the genera frequently micropropagated in that country, with a production of 34 370 plants in 1988 (Pierik 1991).

2 Natural Methods of Bromeliad Multiplication

Bromeliad plants can be propagated through sexual and asexual processes. The sexual process involves seed formation from which large quantities of plantlets can be obtained. The seed is generally small; the seeds of Tillandsioideae are 1 to 7 mm long and the fruits need about 6 to 12 months to mature (Rauh 1990). Viability may be retained for several months. However, the best results of germination were obtained when seeds were sown as soon as possible after harvesting (The Bromeliad Society 1977). Depending on the species, germination takes place in 1 to 3 weeks. In the case of many species of genera such as *Pitcairnia*, *Vriesea*, *Billbergia*, *Aechmea*, and *Neoregelia*, the frequency of germination was high under a variety of light and temperature conditions (Downs 1964; Mercier and Guerreiro 1990).

However, sexual propagation has been demonstrated to have disadvantages, one of the most limiting being the availability of seed. In *Vriesea*, *Tillandsia*, and *Gusmania*, for instance, seeds reach the necessary maturation about 1 year after pollination. Furthermore, the seedlings of these tillandsioids require about 5 to 8 years to reach maturity while, on the other hand, other bromelioid and pitcairnioid plants can be more precocious, flowering in 3–4 years (The Bromeliad Society 1977). In addition to slow growth, tillandsioid seedlings, particularly, are often attacked by pathogens, so that it is recommended that their surface is kept dry at frequent intervals to prevent them becoming overgrown with algae and fungi (Benzing 1980).

Asexual reproduction generates new plants by growing offshoots. This happens in nearly all the bromeliad species after the flowering period. In most cases, the parent plant dies slowly after blooming. When it is on the decline, however, offshoots grow from one or more axillary leaf buds. They can survive independently when they have reached one-third of the size of the parent rosette. Rooting often takes place spontaneously when plants are this size, and it is very easy to detach and plant them in a mixture of equal parts of sand and moss peat. Other species produce their offshoots from a stolon, on which the new rosette grows. It may take root if the stem is covered with the same mixture (Wall 1988). In general, bromeliad species initially produce one to three offshoots, but if the first are removed, more dormant buds are stimulated to develop as the parent plant dies (Wall 1988).

Asexual propagation has the advantages of avoiding the genetic variability inherent in sexual processes, and a shorter time for flowering. In most species offshoots reach flowering maturity sooner than seedlings. This natural method of asexual reproduction, however, is incapable of producing the large numbers of plants needed in commercial nurseries. Furthermore, the space required to produce bromeliads commercially from offshoots would be very expensive. There are exceptions, such as the small *cryptanthus* species, which generally develops many offshoots (The Bromeliad Society 1977).

3 Tissue Culture and Micropagation

In vitro cultures have been used as a tool in the acquisition of knowledge of some physiological characteristics of bromeliads, like nutritional requirements, endogenous hormone production, and other developmental aspects of these plants. Studies performed in vitro have shown interesting nutritional adaptations of epiphytic bromeliads in relation to the growth of seedlings of some epiphytic tank bromeliads and terrestrial nontank types. It was observed that the former showed a higher capacity to metabolize organic nitrogen sources than the latter. According to Benzing (1970), these results reflect the fact that, under natural conditions, the organic nitrogen forms in the water may be the principal nitrogen source. In contrast, the level of inorganic nitrogen would seem to be low in epiphytic habitats. Studies carried out in our laboratory (Mercier 1993; Mercier et al. 1997, in press) showed that bromeliad

seedlings of three different growth habits in nature (terrestrial, tank epiphyte, and atmospheric epiphyte), cultured in vitro with different sources of inorganic and organic nitrogen, showed distinct patterns of endogenous hormonal production (ABA, AIA, Z, ZR, iP, and iPA), polypeptidic and isoenzymatic profiles (GDH and AAT), phenolic compounds, free ammonium ions, and total nitrogen accumulations. In these papers, various aspects of nitrogen metabolism of bromeliads are compared and related to natural nutritional requirements.

In relation to some effects of the culture media and growth regulators on seed germination and seedling development, Mekers (1977) observed that the presence of NAA or GA, at a concentration of 1 mg/l in Knudson basal medium promoted the germination of *Vriesea splendens* at a higher rate than in control plants. Furthermore, NAA stimulated early development of the seedlings. Interesting to highlight is the fact that in the case of *Vriesea hieroglyphica* (Fig. 1a), using a Knudson basal medium in the absence of any growth regulators, from three to seven plantlets were formed per seed, rather than the expected one seedling produced through the normal germination

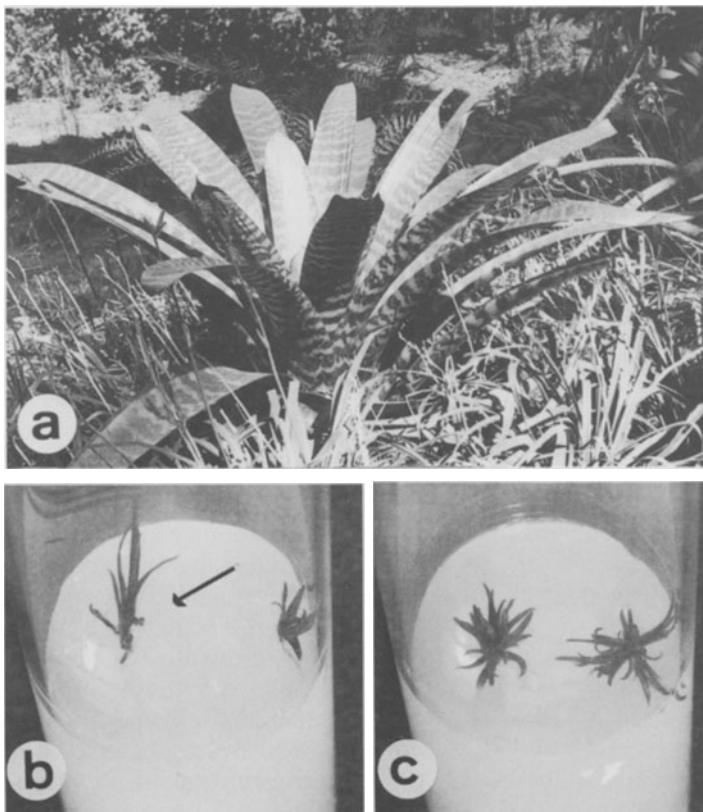


Fig. 1a-c. **a** Field-grown *Vriesea hieroglyphica* plant. **b,c** Early development of *V. hieroglyphica* seedling on Knudson medium. **b** A seedling arising from one seed (arrow). **c** Two clusters of shoots formed from seed

process (Fig. 1b,c). Mercier and Kerbawy (1995) suggested that this result could reflect a case of polyembryony induced by the culture medium.

Pierik et al. (1984), examining the influence of several auxins on in vitro germination and subsequent growth of three different bromeliads, observed that NAA added to a Murashige and Skoog medium was the most stimulating auxin in promoting root and shoot growth. Optimal NAA concentration varied according to the species used, ranging from 0.5 to 0.8 mg/l. A summary of the principal basic studies on bromeliad plants making use of in vitro cultures is presented in Table 1.

During the past 10 years, a considerable number of articles have been published on commercially significant bromeliad micropropagation methods. In 1973, Mapes tried to cultivate some ornamental bromeliads, but with limited success. Jones and Murashige, in 1974, were apparently the first to report a procedure for rapid clonal propagation of *Aechmea fasciata*, one of the most important commercial flowering ornamental bromeliads. Good multiplication rates and high survival were obtained. More recently, in the 1990s, in vitro techniques have been employed to propagate endangered or rare bromeliad species, directly related to conservation programs. A summary of bromeliad micropropagation research is shown in Table 2.

3.1 Disinfection of Explants

Zimmer and Pieper (1976) pointed out that it is difficult to establish bromeliad cultures from shoot tips and axillary buds excised from a field of adult plants. The effectiveness of disinfection can be considered low, as the best results were obtained when the explants were removed from young shoots. Davidson and

Table 1. In vitro basic studies on bromeliads

Type of culture and species used	Main approaches	Reference
Seedling culture of <i>Aechmea bracteata</i> , <i>A. recurvata</i> , <i>Vriesea jonghui</i> , <i>Pitcairnia andreana</i> , and <i>Puya mirabilis</i>	Effect of different nitrogen source on seedling growth and development	Benzing (1970)
Seedling culture of <i>Vriesea splendens</i>	Hormonal requirements for seed germination and early development of seedlings	Mekers (1977)
Seedling cultures of <i>Guzmania minor</i> var. <i>Vela</i> , <i>G. lingulata</i> var. <i>Splendens</i> and <i>Vriesea splendens</i> var. <i>Fire</i>	Effect of naphthaleneacetic acid on seed germination and seedling development	Pierik et al. (1984)
Seedling cultures of <i>Pitcairnia flammea</i> , <i>Vriesea philippocoburgii</i> , and <i>Tillandsia pohliana</i>	Influence of nitrogen source on growth, endogenous hormonal production, polypeptidic and isoenzymic profiles of seedlings	Mercier (1993)
Seedling culture of <i>Vriesea hieroglyphica</i>	Effect of Knudson medium concentration on the growth of seedlings	Mercier and Kerbawy (1994)

Table 2. Summary of the micropagation results with bromeliads

Species	Explant	Shoot regeneration medium	Rooting conditions	Reference
<i>Guzmania</i> sp.	Shoot tips	K (gelled)	Data not given	Mapes (1973)
<i>Ananas erectifolius</i>		20% coconut milk		
<i>Portea petropolitana</i>		Adenine (20)		
<i>Aechmea fasciata</i>	Shoot tips	MS (liquid)	IAA (2) + Kinetin (2)	Jones and Murashige (1974)
	Axillary buds	3%	Ex vitro	
		Adenine sulfate (40)		
		Thiamine-HCl (0.4)		
		i-Inositol (100)		
<i>Aechmea fasciata</i>	Axillary buds	K (liquid)	BAP (1)	Zimmer and Pieper (1976)
<i>Aechmea distichantha</i>	Leaf and stem culture in vitro	2%		
<i>Aechmea</i> (a hybrid)		10% bleeding sap of birch tree		
<i>Guzmania lingulata</i> var. minor	Seeding culture in vitro	K (gelled)	NAA (1) + BAP (1)	Mekers (1977)
<i>Tillandsia polystachya</i>		MS (microelements)	NAA (0.5) + BAP (1)	
<i>Vriesea heliconioides</i>		2%	IAA (1) + BAP (0.5)	
<i>Vriesea splendens</i>		meso-Inositol (100)	NAA (1) + BAP (0.1)	
<i>Vriesea Meyers</i>		Nicotinic acid (5)	NAA (1) + BAP (0.5)	
Favori et		Thiamine-HCl (5)	Pyridoxine-HCl (0.5)	
		Glycine (4)	Glycine (4)	
		Adenine (40)	Adenine (40)	
<i>Cryptanthus bivittatus</i> minor	Axillary buds	MS (liquid)	IAA (2) + Kinetin (2)	MS (gelled) + NAA (1 mg/l) + Kinetin (2 mg/l)
		3%		Davidson and Donnan (1977)
		Thiamine-HCl (0.4)		
		i-Inositol (100)		
		Adenine sulfate (80)		

<i>Quesnelia quesneliana</i>	Axillary buds Leaf culture In vitro	MS (liquid) 2%	NAA (1) + BAP (1)	MS (gelled) + NAA (0.1 mg/l)	Hosoki and Asahira (1980)
<i>Vriesea poelmanni</i>	Axillary buds	MS (gelled)	NAA (2) + IBA (2) Kinetin (2)	MS half-strength	Mathews and Rao (1982)
<i>Aechmea fasciata</i>			NAA(0.1) + BA (2)	VW (gelled) + NAA (1 mg/l) + IBA (1 mg/l) + 0.2% activated charcoal	Rogers (1984)
<i>Cryptanthus bromelioides</i> var. tricolor	Axillary buds	VW (gelled) 2%	NAA (0.005) + BAP (0.2)		
<i>Tillandsia dyeriana</i>	Seedling culture in vitro	Nicotinic acid (1) Thiamine-HCl (10) Pyridoxine-HCl (1) myo-Inositol (100) Bacto-Peptone (2)			
<i>Tillandsia cyanea</i> var. Anita	Shoot tips	10% coconut water MS (liquid) NaFeEDTA-25mg/l 3.5%	NAA (0.005) + BAP (0.2)	MS (gelled) + NAA (0.8 mg/l)	Pierik and Sprengels (1991)
<i>Vriesea fosteriana</i>	Seedling culture in vitro	Nicotinic acid (5) Vitamin B1 (5) Pyridoxine (0.5) Glycine (4) meso-Inositol (100) K (gelled and subsequent transfers to liquid) MS (microelements) 2%	NAA (0.5) + BAP (2)	K (gelled) + MS (microelements) + NAA (0.2 mg/l)	Mercier and Kerbau (1992)
<i>Puya tuberosa</i>	Segments of immature leaf	MS (gelled) 3%	NAA (0.1-0.5) + BAP (5-10)	Directly on shoot regeneration medium	Varadarajan et al. (1993)
		Nicotinic acid (0.5) Thiamine HCl (0.5) Pyridoxine (0.5) Glycine (2)			

Table 2. Continued

Species	Explant	Shoot regeneration medium		Rooting conditions	Reference
		Basal medium ^a , % sucrose, and organic supplements (mg/l)	Growth regulators (mg/l)		
<i>Dyckia macedoi</i>	Leaf culture in vitro	K (gelled) 2%	NAA (0.1) + BAP (5)	Directly on shoot regeneration medium	Mercier and Kerbauy (1993)
	Seedling culture in vitro	K (gelled) 2%	NAA (0.5) + BAP (2)	K (gelled) + IBA (5.0mg/l)	
<i>Vriesea heteroglyphica</i>				MS (gelled) + IBA (0.2–2.0mg/l or NAA (0.1–1.0mg/l)	Mercier and Kerbauy (1994, 1995)
<i>Acchmea fasciata</i>	Stem Leaf	MS (gelled) 2% Inositol (100)	IAA (1) + Kinetin (1)	MS (gelled) + IBA (0.2–2.0mg/l or NAA (0.1–1.0mg/l)	Vinterhalter and Vinterhalter (1994)
	Glycine (2) Nicotinic acid (0.5) Thiamine-HCl (0.4) Pyridoxine-HCl (0.5)				

^aMS = Murashige and Skoog (1962); K = Knudson (1946); VW = Vacin and Went (1949).

Abbreviations: BAP = 6-benzylamino purine; IAA = indole-3-acetic acid; IBA = naphthalene acetic acid.

Donnan (1977) recommended keeping mother plants of *Cryptanthus bivittatus* minor in a dry air-conditioned room for 2–4 weeks before removing the buds. This procedure reduced the percentage loss in the establishment stage.

The most commonly employed surface disinfectant has been sodium hypochlorite, which is generally applied as a diluted solution (1–20%) with a few drops of a wetting agent added, such as Tween 20. Different times have been used (5–30 min). In every case the explants were washed several times with sterile distilled water and then transferred to the media. Effective surface disinfection of lateral buds of *Cryptanthus bromelioides* var. tricolor with 0.1% mercury chloride for 10 min was also reported (Mathews and Rao 1982).

3.2 Media Composition

In general, the basal nutrient media used for bromeliad micropropagation have been those proposed by Murashige and Skoog (1962) and Knudson (1946), in gelled or liquid state at full or reduced strength. Organic components such as vitamins and carbohydrates are often used at various concentrations.

Combinations of the growth substances BAP and NAA have generally been recognized as the most efficient way to induce adventitious bud formation or axillary branching. However, depending on the species, other combinations such as IAA and BAP (Mekers 1977), IAA, and kinetin (Jones and Murashige 1974; Vinterhalter and Vinterhalter 1994), NAA, IBA, and kinetin (Mathews and Rao 1982) have also been employed with success.

The elongation stage of regenerated shoots is considered by some authors as the most responsive phase for inducing adventitious rooting. In order to stimulate the apical growth of the new leafy shoots, an auxin is used, generally NAA, at low concentration. In some cases, the rooting was achieved by excluding growth regulators from the medium, as observed with *Aechmea fasciata*, *Aechmea distichantha* (Zimmer and Pieper 1976) and *Cryptanthus bromelioides* var. tricolor (Mathews and Rao 1982). In this latter plant the MS medium was reduced to half. However, NAA, at several concentrations, was used in most of the studies on bromeliad root development (Hosoki and Asahira 1980; Pierik and Sprenkels 1991; Mercier and Kerbauy 1992, 1993). Mekers (1977), however, observed that IAA was the most suitable auxin for the rooting process of *Tillandsia polystachya*. Vinterhalter and Vinterhalter (1994) showed that the length of the roots increased significantly upon addition of 1% activated charcoal to the media supplemented with various concentrations of NAA or IBA or if sucrose was replaced by equimolar glucose and fructose. Recent results obtained in our laboratory with *Vriesea hieroglyphica* showed that 5 mg/l IBA was able to promote root development (Mercier and Kerbauy 1995).

3.3 Shoot Tip, Axillary Bud and Leaf Explants from Adult Bromeliads

Mapes (1973), based on the results with pineapple cultures, tried to multiply seven ornamental bromeliads. From these, only three species: *Portea*

petropolitana, *Guzmania* sp. and *Ananas erectifolius* showed positive response in modified Knudson medium with 20% coconut milk and adenine. This medium stimulated the development of lateral buds and also the induction and growth of globular bodies (protocorm-like bodies), which subsequently developed into plantlets.

After 6 months in culture, an average of 500 regenerated plantlets from each shoot tip and axillary bud of *Aechmea fasciata* was reported by Jones and Murashige (1974). Between 90 and 100% of these new shoots survived, giving rise to phenotypically normal plants. After ca. 3 months of culture, Hosoki and Asahira (1980) obtained four to eight adventitious buds from each lateral bud of *Aechmea fasciata*, *Quesnelia quesneliana*, and *Vriesea poellmannii*. The plantlets formed from these adventitious buds were successfully established and developed normally.

The successful production of *Cryptanthus bivittatus* minor was achieved when axillary buds were cultured in a multiplication medium with induced callus formation prior to shoot development. From one mother plant 10000 new bromeliads can be produced after 9 months of in vitro culture. The loss in the transplanting phase was very low and the plants presented normal pink-brown coloration in the growing areas (Davidson and Donnan 1977). Mathews and Rao (1982) observed a chimeric segregation in *Cryptanthus bromelioides* var. tricolor when lateral buds excised from adult plants were used. The adventitious shoots developed into plants of three different color patterns: striated (comparable to the natural type), entirely green, and yellowish white. Only the green plants could be transplanted to soil, therefore, with up to 90% survival. Interestingly, it was observed that after being established in the soil, some of the green plants still underwent color changes, resulting in 20% reddish purple and 30% green and purple stripes, while 50% retained their original green color.

Another ornamental bromeliad micropropagated by shoot tips was *Tillandsia cyanea* cv. Anita (Pierik and Sprenkels 1991). In this case, axillary-formed shoots of plants grown in a greenhouse were used as initial explants. In spite of the rigorous disinfection process used by these authors, only a reduced number of totally sterile shoots was obtained. Shoot multiplication took place by axillary branching and an average of 14.6 new plantlets formed after 10 weeks of culture. Pierik and Sprenkels (1991) also observed that the plants could easily be transferred to soil, and thereafter grew and flowered normally.

A more recent method was reported by Vinterhalter and Vinterhalter (1994) for large-scale commercial propagation of *Aechmea fasciata* Baker, which completely avoids or eliminates aberrant plant formation.

3.4 Seedlings and Young Leaf Explants

Seedling culture, obtained through in vitro seed germination, is also used as a worthwhile strategy for establishing bromeliad culture. The great number of aseptic donor explant seedlings obtained by this method means that practically

all parts of seedling can be used as explant sources. For example, the prominent regenerative expression of young leaves found in some species can be more easily explored. Furthermore, the seedlings themselves can give rise to new shoots. Both seedlings and young leaves have routinely been used in our laboratory as a remarkable source of explants in our Brazilian bromeliad conservation program. In the case of endangered and/or rare species, this method has proved to be of great value, since it is very difficult to obtain a large number of mother plants.

Obviously, the most important advantage in using these sources of explants is the possibility of totally overcoming the recognized difficulties of disinfecting shoot tips and axillary buds excised from grown field plants.

Axillary shoot development of some bromeliads of the Tillandsioideae subfamily was obtained from in vitro seedling cultures (Mekers 1977; Rogers 1984; Mercier and Kerbaul 1994). For *Vriesea hieroglyphica*, the multiplication rate was ca. seven shoots per plantlet after 6 months of culture (Mercier and Kerbaul 1994). However, in *Vriesea fosteriana* (Fig. 2a) seedlings, Mercier and Kerbaul (1992) observed only adventitious bud formation, which took place from protuberances (masses of cells) which arose at a position corresponding to the second internode (Fig. 2b). The best proliferation rate was 22.5 buds per seedling after 3 months of culture on gelled Knudson medium to which was added $2.7\mu\text{M}$ NAA and $8.9\mu\text{M}$ BA. After subsequent transfer, the number of buds doubled when the same medium was used in a liquid state, and a further increase of 20% in number of buds was achieved after a second transference to a liquid medium with the same composition. The addition of NAA ($0.54\mu\text{M}$) to a gelled Knudson medium resulted in inhibited adventitious proliferation, and apical shoot growth was reestablished. (Fig. 2c). When the shoots reached 2 cm in height, rooting was induced; separate shoots were transferred to a medium supplemented with $1.1\mu\text{M}$ NAA. After 2 months of culture each shoot formed three to four roots (Fig. 2e). The plantlets were transferred to collective pots and kept in a greenhouse under natural day conditions (Fig. 2f). After 1 year, 100% of the plantlets survived and looked phenotypically normal. They could be transferred to wild conditions without any problems.

Young leaves detached from aseptic cultured seedlings of *Vriesea fosteriana* and *Dyckia macedoi* (Mercier and Kerbaul 1992, 1993) produced adventitious buds on their basal parts. In *Dyckia macedoi*, buds were formed both directly from leaf tissue and from protuberances after nearly 2 months of culture, while in *Vriesea fosteriana*, about 15 buds were formed only through protuberances after 1 month of culture on gelled Knudson medium containing $2.7\mu\text{M}$ NAA and $8.9\mu\text{M}$ BA (Fig. 2d). The number of leaf-derived buds can be duplicated by means of a subculture into a liquid medium of the same composition. Similar base leaf regeneration in bromeliads was also observed on the base of leaf sheaths removed from greenhouse-grown seedlings of *Puya tuberosa* (Varadarajan et al. 1993).

Like the seedlings germinated in vitro, the plantlets regenerated from shoot tip and axillary buds proved to be an important source of young leaf explants. The results presented by Hosoki and Asahira (1980) showed the

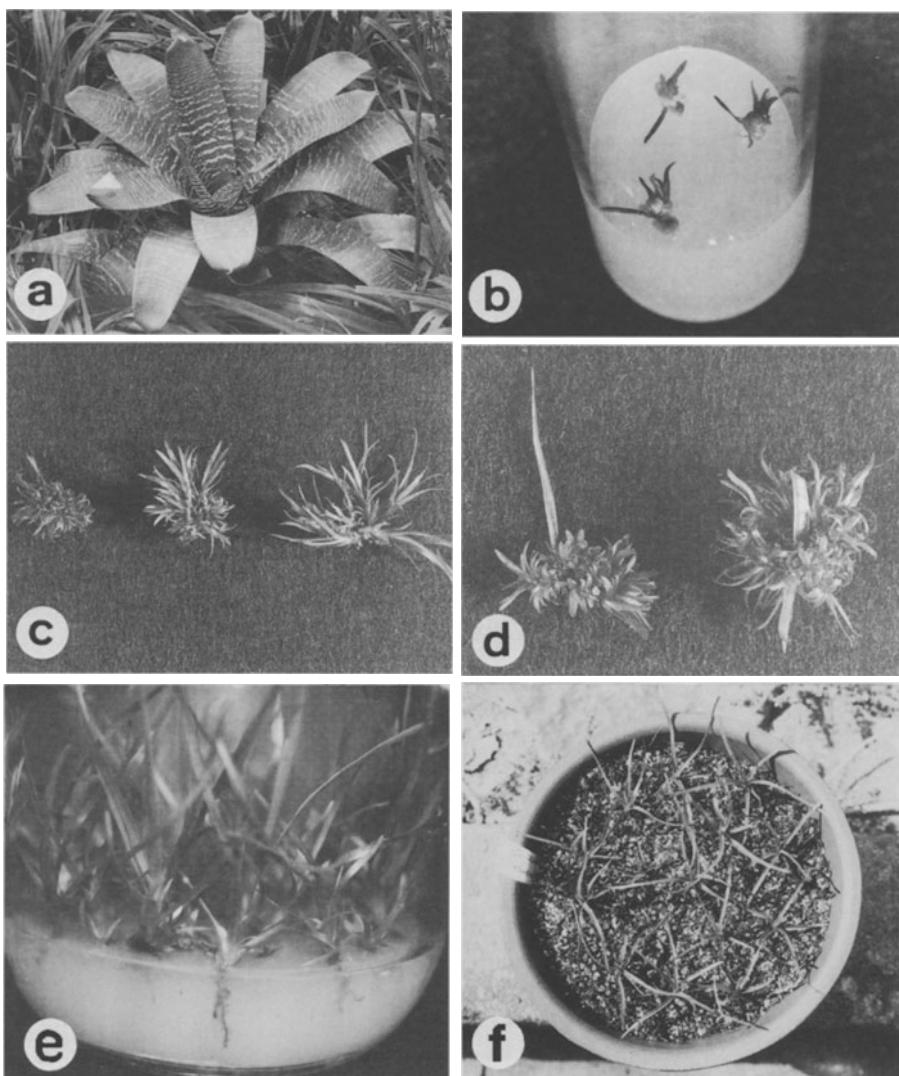


Fig. 2a-f. **a** Field-grown *Vriesea fosteriana* plant. **b** Seedlings of *V. fosteriana* showing protuberances being formed at their base. **c,d** Clusters of *V. fosteriana* shoots formed from both seedling (c), and from leaf explants (d). **e** Rooted plantlets of *V. fosteriana* 4 weeks after transfer onto the rooting medium enriched with $1.1 \mu\text{M}$ NAA. **f** In vitro regenerated plants of *V. fosteriana* after transfer to collective pots

formation of protuberances at the basal part of the leaf tissues of *Aechmea fasciata*, *Quesnelia quesneliana*, and *Vriesea poelmannii*. After 30 days of culture, buds were formed from these protuberances, producing a mean number of 6, 10.5, and 10 buds per leaf, respectively. The high regenerative potential of the leaf base was attributed by these authors to the presence of intercalary meristems found in this region.

3.5 Genetic Stability

Some variability has been observed in regenerated plants of *Aechmea fasciata* obtained from shoot tip and axillary bud explants. Jones and Murashige (1974) reported the occurrence of genetically changed plants reaching 20% in prolonged in vitro culture. Long-term callus cultures seem to be deleterious to genetic stability of bromeliads. Zimmer and Pieper (1976) found a rate of over 50% of aberrant plants with an *Aechmea* hybrid after 1 year of culture. The modifications were observed mainly in leaf number, position, color, and size. These authors supposed that a great number of these plants were of chimeric constitution. According to Ziv et al. (1986), some of the *Aechmea fasciata* variant plants regenerated in vitro reverted back to their normal pattern in the second year of growth in the greenhouse, implying that the change was epigenetic. Vinterhalter and Vinterhalter (1994) showed that callus of *Aechmea fasciata* can be used in the initial propagation stage in order to obtain a number of shoots. Once the shoot cultures are established, they can be subcultured on a medium containing a certain hormone combination that ensures a sufficient propagation rate and prevents aberrations.

In spite of the occurrence of somaclonal variation indicated above, bromeliad plants obtained from seedlings and young leaf tissues showed no genetic alterations. They showed phenotype fidelity, this fact being of great importance to the germplasm repository program, as well as in commercial practices.

4 Summary and Conclusions

Ornamental bromeliad plants are of increasing economic importance in many countries. The development of micropropagation methods has allowed efficient exploitation of several decorative species, since natural vegetative multiplication can be considered very slow, producing very few shoots per plant.

Successful plantlet formation has been achieved through shoot tips, axillary buds and leaves removed from adult plants. There are also reliable methods for seedling cultures, which can be considered of great importance for preserving the germplasm of threatened bromeliads, assuring the natural variability of a given species. Basal parts of leaves removed from aseptic cultured seedlings gave rise to a great number of adventitious buds. The plantlets formed showed no alterations in morphology or in pigmentation patterns. Besides this, bromeliad plants formed in vitro showed no difficulties in acclimatizing when transferred to pots.

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I.5 Micropropagation of *Campanula*

K. BRANDT¹

1 Introduction

1.1 The Plant and Its Importance

The genus *Campanula* comprises approximately 300 species distributed across the Northern Hemisphere (Cook 1951), many of these in mountainous areas. The genus generally inhabits meadow and subalpine regions, many species requiring full sun for optimal development. All species are herbaceous, and the name refers to the blue, bell-shaped flowers of the majority of the species. They are perennials, biannuals, or annuals. The growth pattern is characterized by a distinct vegetative phase, often with development of rosettes, and a generative phase, induced by long photoperiods, vernalization, or both, where the rosettes develop into multi- or single-flowering inflorescences. Usually, a flowering shoot dies after seed maturation. In biannual and annual species each seedling usually forms one rosette. The perennial species develop sideshoots or more or less underground runners each of which becomes a rosette during the following growth season.

Many species are used by alpine gardening enthusiasts, and a number of species are grown commercially on a larger scale. Some of the larger species, e.g., *Campanula medium* and *C. pyramidalis*, have traditionally been used as cut flowers, and a number of smaller species are grown as pot plants. The species *Campanula isophylla* from the Italian Alps is a traditional pot plant in several countries. In recent years, other species, among them the larger-flowering *C. carpatica* or the miniatures *C. portenschlagiana* and *C. cochlearifolia*, have gained increasing importance. The Danish production of *Campanula* pot plants was in the order of 6 million plants in 1994. Most of these plants are *C. carpatica* or other hardy species, grown in so-called combined production systems. In these, the plants are grown in pots or as plugs outdoors after propagation and taken into the greenhouse for flower development after Christmas.

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Fig. 1. Flowering plant of *Campanula isophylla*

1.2 Commercial Propagation and Cultivation

Traditional propagation is either by seed or cuttings; biannual species, such as *Campanula medium* and *C. pyramidalis*, are seed-propagated, while the perennial species may be vegetatively propagated by cuttings or runners during the vegetative phase. If the plants enter the generative phase, the shoots rapidly lose the ability to form roots (Moe and Andersen 1988).

This chapter concentrates on *Campanula isophylla* and *C. carpatica*, which are marketed as pot plants grown in 10-cm pots. The two species superficially look alike, appearing as a hemisphere 20–30 cm in diameter, covered with blue or white star-shaped flowers. However, the cultivation procedures are different.

C. isophylla is sensitive to frost, so vegetatively propagated cultivars are grown in greenhouses. Flower induction is controlled by photoperiod, long days being inductive. Temperature has only a minor influence; by modifying the critical daylength, a linear relationship has been found in the range from 12°C/16h to 21°C/13h (Heide 1965). The species shows no dormancy or vernalization requirements, and it is therefore relatively easy to keep the plants growing vegetatively, simply by keeping the photoperiod shorter than 12h. This species is usually propagated by cuttings taken from the relatively open rosettes. In recent years, several companies have marketed seed-propagated cultivars.

In *C. carpatica*, vegetative propagation is more complicated, since the plants enter dormancy after flowering. A period of vernalization is necessary for resumption of vegetative growth, and specifically promotes the formation of runners from the base of the plant; they grow underground and surface 2–10 cm from the stock plant. The flowering process is then induced by long photoperiods (Kristiansen 1988). Seedlings can flower without vernalization, but then several seedlings in a pot are necessary. Some cultivars are grown

from seed, but since the stock plants can be grown outdoors at moderate expense, vegetatively propagated cultivars are also common. In spring, the plants are divided into single shoots, that will each grow into one plant during the summer. A typical stock plant will produce 10–20 runners a year.

1.3 Need for Micropropagation

For perennial species which can be vegetatively propagated by cuttings or runners, conventional vegetative propagation can be used to produce plants for sale at a lower price than is possible by micropropagation. However, there are three main purposes in micropropagation of *Campanula*.

1.3.1 Reduced Risk of Contaminating Stock Plants with Fungal Diseases

A number of fungal diseases have been recognized in *Campanula* (Mygind 1986; Thinggaard 1994). In *C. isophylla* and *C. poscharskyana* micropropagation has been used as a way of obtaining healthy plant material and/or preserving selected genotypes as stock plants for vegetative propagation (Ó Ríordáin 1994; N. Paludan and H. Hildrum, pers. comm.) or breeding (S. Ringgaard, pers. comm.).

In this case micropropagation is at the level of nuclear stock plants.

1.3.2 Higher Propagation Rates than Traditional Vegetative Propagation

Particularly in *Campanula carpatica*, the introduction of new vegetatively propagated cultivars creates a demand for rapid propagation to build up a sufficient stock before production of plants for sale can start (S. Ringgaard, pers. comm.). Micropropagation is at the level of second generation stock plants, the immediate mother plants of the marketed plants.

1.3.3 Vegetative Propagation of Biannual Species

In *C. pyramidalis*, micropropagation allows for vegetative propagation of selected clones (D. Holdgate and R. Theiler-Hedtrich, pers. comm.), since seedlings are rather variable and in vivo clonal propagation is difficult due to the biannual growth cycle of this species (Zimmer 1983). In this species the micropropagated plants are grown directly into the final product.

2 Micropropagation

Only very few reports exist on micropropagation of *Campanula*. Fahrenkrog (1983) conducted studies on micropropagation of *Campanula pyramidalis* by adventitious shoot formation from petioles; however, the shoots formed were

not viable. Micropagation of *C. isophylla* has been described by Brandt (1992, 1994). In the 1994 COST 87 Directory of European Plant Tissue Culture Laboratories (Ó Riordáin 1994), four laboratories report work on a total of four species of *Campanula*: *carpatica*, *isophylla*, *poscharskyana*, and *pyramidalis*. One commercial laboratory has propagated *Campanula pyramidalis* by regeneration from cell cultures, with technical but not commercial success (D. Holdgate, pers. comm.), while micropagation by shoot cultures has been a more viable procedure (R. Theiler-Hedtrich, pers. comm.).

Most of the experiments described below were made with *Campanula isophylla*, and the resulting procedures used for *C. carpatica*. For this reason, the description refers generally to *C. isophylla*, but can also be used for *C. carpatica*. Cases where *C. carpatica* reacts differently or where specific experiments have been made with this species are specified. A micropagation procedure developed independently for *C. pyramidalis* (R. Theiler-Hedtrich, pers. comm.) is essentially identical to the one described for *C. isophylla*.

2.1 Establishment of Axillary Shoot Culture

Vegetative shoots of ca. 10cm length were defoliated, leaving approximately 1cm of the petioles, and predisinfected in 1% Korsolin (Ferrosan, a disinfection agent containing 3.8% formaldehyde, 8% glutaraldehyde, and ethylene glycol) for 10min. The shoots were cut in 1-cm segments with at least one axillary or apical bud and the petioles shortened to 5mm. The segments were disinfected in 3% Korsolin for 5min and rinsed in sterile deionized water, and internodes and petioles were further trimmed to reach the desired explant size. Stem segments were transferred to 100 × 30 mm flat-bottomed glass tubes containing 15 ml MS medium (Murashige and Skoog 1962) with the macronutrients reduced to 50%, 30 g/l sucrose, 4.4 µM benzyladenine (BA) and 6 g/l

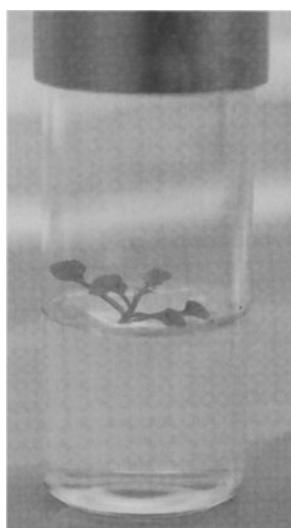


Fig. 2. Shoot from vegetative explant of *C. isophylla*

agar (Difco Bacto). The pH was adjusted to 5.8 with KOH before autoclaving for 20 min at 121 °C. Cultures were kept at 22 ± 3 °C and supplied with cool white (20 µmol/m²/s) fluorescent light (16 h/day). For *C. carpatica* 12 g/l agar was used, and a photoperiod of 12 h.

Three explant sizes were compared for culture initiation: 1-, 1.5-, and 2-mm stem segments. Size of the primary explant significantly influenced the formation of the initial shoot, since the chance of shoot formation was reduced in small explants ($p < 0.001$) while the risk of contamination was not affected (Table 1). Shoot formation also depended on genotype ($p = 0.004$).

Contamination can be a problem in *Campanula*, because the best (most vegetative) explants are found close to the soil surface, and thus are often heavily infested with soil microorganisms.

If generative buds are used as explants, they continue their differentiation into small inflorescences, often with the development of flowers in vitro. Some of these generative plantlets will eventually develop vegetative side shoots, but delayed in comparison with vegetative explants. Due to a greater distance from the soil surface, and a tendency for systemic *Fusarium* infections to stay in the basal part of the plants (Mygind 1986), it may be an advantage to use generative shoots if contamination is a major problem.

Table 1. Percentage of primary explants of different sizes that were contaminated and percentage of uncontaminated explants that produced shoots. LSD_{0.95} is given for a comparison of two explant sizes. (Brandt 1992)

Size (mm)	Contamination (%)	Shoots formed (%)
1	8.4	57.8
1.5	8.6	72.6
2	10.1	85.1
LSD _{0.95}	7.2	7.3

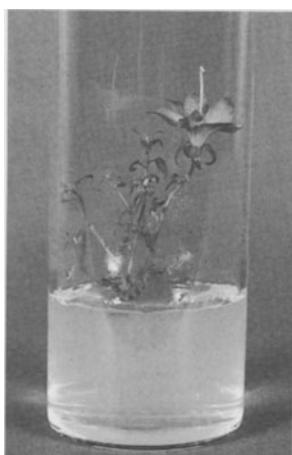


Fig. 3. Shoot from generative explant of *C. isophylla*

Development of flowers of *Campanula* in vitro is currently being used in our laboratory as a model system to investigate the effects of environment and genotype on flower development, in particular anthocyanin synthesis. (Brandt and Ishimaru 1997).

2.2 Formation and Division of Shoot Clusters

After 5 weeks on initiation medium, the explants were moved to 100×38 mm tubes with 20 ml full-strength MS medium and $4.4 \mu\text{M}$ BA. Subsequently, every 5 weeks all shoots were excised 3 mm above the basal part of the shoot cluster. In the statistical analyses transformed values of numbers of shoots and roots were used, $\ln(\text{shoot number})$ and $\ln(\text{root number} + 1)$, respectively, since the standard deviation was otherwise proportional to the average values, and since many plants did not form any roots on propagation medium. Values of number of shoots or number of roots shown in Figs. 4, 7–10 are back-transformed from the logarithmic averages, and the least significant difference between means (LSD) was backtransformed to the least significant quotient (LSQ). For analysis of variance and correlations the general linear models procedure (GLM) (SAS Institute 1989) was used.

Callus, leaves, and brown tissue were removed. The basal part was divided vertically, and sections, each consisting of four shoot bases, were transferred to fresh medium. Numbers of shoots and roots, plant height (height of the entire shoot cluster) and dry weight of excised shoots were recorded at transfer.

The number of new shoots per shoot cluster at each subculture increased during the first seven subcultures, then leveled off (Fig. 4). On the other hand, the small but significant changes in the number of roots per shoot cluster and the height of the plants did not show any consistent patterns (Fig. 4).

The excised shoots were used for root induction (see Sect. 2.3). They could also be used to initiate new shoot clusters, but a comparison of three kinds of explants taken from a shoot cluster (basal sections, nodes and shoot tips)

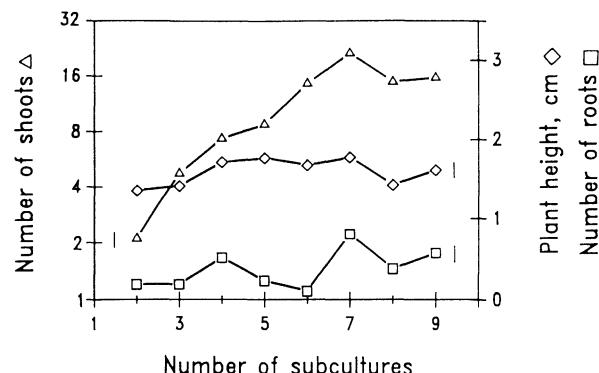


Fig. 4. Plant height and average number of shoots and roots at the end of each subculture for 29 clones. Bars denote $\text{LSD}_{0.95}$ for plant height, $\text{LSQ}_{0.95}$ for numbers of shoots, and $\text{LSQ}_{0.95}$ between two levels of (number of roots + 1). (Brandt 1992)

Table 2. Plant height, callus formation (shoot clusters showing any soft callus), and number of roots and shoots per shoot cluster developed from three kinds of explants. LSD_{0.95} is given for plant height and callus formation, for shoot numbers the value is LSQ_{0.95} and for root numbers it is LSQ_{0.95} between two levels of (number of roots +1). (Brandt 1992)

Explant type	Plant height (mm)	Callus formation (%)	No. of roots	No. of shoots
Basal section	17.3	65.0	0.35	8.7
Node	8.3	—	—	1.5
Shoot tip	8.0	25.0	0.26	2.1
LSD, LSQ	3.9	35.2	1.41	1.8

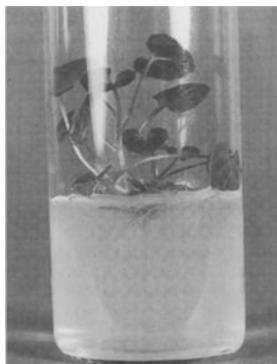


Fig. 5. Rooted shoot of *C. isophylla*

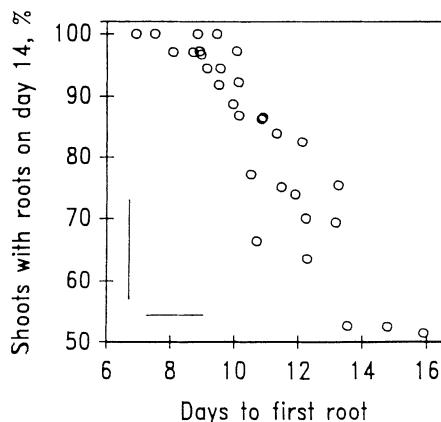
showed the basal sections to give the best results (Table 2). Reducing apical dominance by removing the apical part of each shoot seems to be important for promoting proliferation of new shoots.

2.3 Root Formation and Ex Vitro Performance

Campanula isophylla. To promote root formation, excised shoots from shoot clusters were grown on rooting medium: MS without growth regulators and with the macronutrients reduced to 50%. The height of each shoot was recorded the day after placing the shoot on rooting medium. Twice a week the number of roots on each shoot was recorded, and from these data the number of roots 20 days after excision of the shoot was calculated, using the nonlinear regression procedure (NLIN) of Statistical Analysis System (SAS Institute 1989) to a logistic curve, separately for each shoot. This rooting experiment was performed at subcultures 9 and 10 with 20 shoots per clone per subculture, using clones from family *a* (see Sect. 2.6 Effects of Genotype).

On rooting medium the majority of the explants of all tested genotypes produced roots within 2 weeks, but the speed of root formation differed significantly (Fig. 7).

Fig. 6. Average number of days to first root for 30 genotypes on rooting medium (including only shoots that formed roots within 30 days), and percentage of plants with roots after 14 days. Bars denote LSD_{0.95}. On day 30, 93.4% had roots. (Brandt 1992)



After 3 weeks on rooting medium, five plantlets per genotype, selected at random among plantlets with at least one root, were transferred to a fertilized peat (Pindstrup 3) in 10-cm pots in the greenhouse. The plantlets were protected from desiccation by a white plastic tent for 10 days after transplantation. Plants were grown at a minimum temperature of 18°C and a photoperiod of 16h to induce flowering, and the number of days to flowering was recorded. When in flower, plants were visually inspected for intraclonal variation in growth habit and in shape and color of flowers and leaves. For nine of the clones data on the number of days to flowering for cuttings, and photographs of conventionally propagated plants, were available for comparison.

Survival of the rooted plantlets after transfer to soil was 80–100% in each clone. Most losses seemed to be due to attack from larvae of fungus gnats (Sciaridae).

No intraclonal variation in qualitative characters was detected, either among micropropagated plants or between micropropagated and cutting-propagated plants. Average number of days from planting in soil to flowering was 74 and 93.4, respectively. Clonal averages of days to flowering was correlated between micropropagated and cutting-propagated plants, $p = 0.036$, $R = 0.70$.

The observed correlation of flowering dates between micropropagated and cutting-propagated plants shows that interclonal differences are preserved. The difference in average time to flowering probably reflects the 21 days that the micropropagated plants spent on rooting medium before transplantation (apart from possible differences in growth conditions). Possibly the setback during acclimatization is equivalent to that which conventional cuttings experience during the rooting period.

Often, it is more difficult to induce root formation in *Campanula carpatica*. A particularly difficult clone was used for an experiment to optimize the rooting medium for this species.

Five different rooting media were compared, each with 45 shoots. After 5 weeks, the number of roots on each plant was recorded. A modified rooting

Table 3. Root formation and survival for shoots of one clone of *C. carpatica* after 5 weeks on different media, 45 shoots per treatment. (Brandt, unpubl.)

Sucrose (%)	Addition to medium	Rooted plants (%)	Dead plants (%)	No. of roots per rooted plant
3	–	22	18	2.5
3	Charcoal	24	27	2.3
3	NAA	29	7	1.8
6	–	31	27	2.2
6	Charcoal	64	16	2.3

medium with 6% sucrose and addition of 0.5 g/l activated charcoal gave the best ($p < 0.0001$) rooting percentage (Table 3). No significant effects of media were found on survival or on the number of roots in shoots that did form roots. To prevent losses due to fungus gnats and fungi during and after the acclimation period, the peat moss was heat-treated (80°C overnight) before transplantation.

Several thousand plants from more than ten clones of *C. carpatica* have been grown to flowering ex vitro. After the 1st year the plants were indistinguishable from traditionally propagated plants of the same clones. Concerning temperature response, plantlets correspond to seedlings; vernalization is not necessary for flower induction in the 1st year ex vitro.

2.4 Effects of Growth Regulators

Basal sections from shoot clusters grown previously on propagation medium were grown for one subculture on factorial combinations of NAA (0 and 0.54 μ M) and BA (0, 4.4, and 8.8 μ M).

The interactions between BA and NAA were not significant, so their effects are represented separately in Table 4. Increasing concentrations of BA gave more shoots, but shorter plants, and fewer roots, while NAA promoted callus formation (Table 4). These effects were all significant at levels higher than 97%. Shoot number and plant height differed among clones ($p = 0.029$ and $p = 0.039$ respectively), but the design of this experiment did not permit an analysis of interaction between clones and treatments.

The roots formed on media with NAA were generally shorter and thicker than on other media. In *C. carpatica*, no promotion of root induction was found, and the inhibition of root elongation combined with excessive callus formation eliminated any positive effect of NAA on the number of roots in those shoots that did form roots (Table 3).

The effects of growth regulators in the media corresponded qualitatively to what has been observed in petiole cultures of *C. pyramidalis* (Fahrenkrog 1983). The lowest concentrations of growth regulators giving adequate number of well-sized shoots (4.4 μ M BA and no auxin) were used as standard for propagation. It is likely that some genotypes would have a different optimum (Fahrenkrog 1983) but the described medium is suitable as a starting point for all the genotypes tested.

Table 4. Plant height, callus formation (shoot clusters showing any soft callus), and numbers of roots and shoots per shoot cluster on media with different concentrations of BA and NAA. LSD_{0.95} is given for plant height and callus formation, for shoot numbers the value is LSQ_{0.95} and for root numbers it is LSQ_{0.95} between two levels of (number of roots +1). (Brandt 1992)

Growth regulator (μM)	Plant height (mm)	Callus formation (%)	No. of roots	No. of shoots
BA				
0	24.1	66.7	6.14	4.8
4.4	18.5	74.4	0.70	9.5
8.8	18.6	64.1	0.55	12.3
LSD, LSQ	3.4	21.4	1.41	1.3
NAA				
0	18.5	46.7	1.05	8.6
0.54	22.3	91.2	2.51	7.9
LSD, LSQ	2.8	17.5	1.32	1.2

2.5 Effects of Light

Basal sections from shoot clusters grown previously at an irradiance of 20 μmol/m²/s were grown for one subculture at 1.7, 4, 9, and 20 μmol/m²/s.

Reduced irradiance caused a reduction in leaf size ($p < 0.001$) and dry weight per shoot ($p = 0.001$), but not in the number of shoots formed per shoot cluster (Fig. 7). Leaf width and dry weight of shoots were largest at the highest irradiance, indicating that the size of shoots may possibly be increased further by increasing the irradiance (Fig. 8). Most of the shoots that develop in one subculture are visible within one week. It is likely that the initiation of new shoots is determined by the growth conditions during the previous subculture. The results correspond well to results for *C. pyramidalis* (Fahrenkrog 1983), where shoot growth was promoted by light while the initiation of adventitious shoots was greater in darkness.

2.6 Effects of Genotype

Among plants grown in vitro, it is often observed that some individuals have a few large shoots that root easily, while other produce clusters of many small shoots that are difficult to root. This is obvious when comparing different treatments, e.g., with light or growth regulators, but also genotype influences the growth habit in vitro. Such differences in number of roots and shoots among genotypes during in vitro culture may be mediated by differences in the endogenous balance of auxin and cytokinin. If this were a common phenomenon, numbers of shoots and roots should generally be negatively correlated among genotypes, and it would be difficult to breed for simultaneous improvements. To investigate if such a negative correlation is present in *Campanula*, the in vitro ability to form shoots and roots was recorded in a population large enough for a statistical analysis (Brandt 1994).

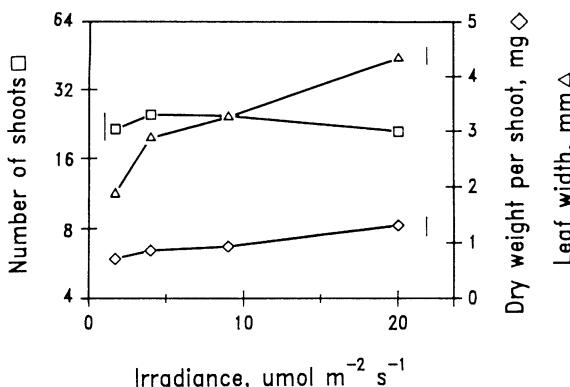


Fig. 7. Number and dry weight of shoots and width of leaves on shoot clusters grown for one subculture at different irradiances. At the previous subcultures all plants were grown at $20 \mu\text{mol}/\text{m}^2/\text{s}$. Bars denote $\text{LSD}_{0.95}$, except $\text{LSQ}_{0.95}$ for number of shoots. (Brandt 1992)

The plants used in the experiments were 20 and 75 seedlings, respectively, from two F_2 families (*a* and *b*), each family was the offspring of two sib-mated F_1 plants. The parent lines of the two sets of F_1 plants were different, and each set had one parent line known to produce many shoots in vitro and one producing fewer shoots. The plants originated from a breeding program at the seed company L. Dæhnefeldt A/S, Marslev, Denmark.

Deviation from clonal mean (DCM) was calculated as measured value for a plant minus the clonal mean (CM) for the corresponding clone. This means that DCM represents the nongenetic, intraclonal variation, as opposed to CM, which contains the genetic component of the variation. To facilitate visualization, DCM was presented in figures by sorting the data into groups (of 20 or 40) according to the x value and computing the average of the y and x values for each group. Calculation of correlations was done on the entire dataset. The combined significance of the observed correlations was found by multiplication of the significances for each family.

The effect of genotype on development in vitro was highly significant, in each family $p < 0.0003$ for number of shoots and roots per shoot cluster, height of shoot clusters, dry weight of excised shoots, dry weight per shoot, height of excised shoots, and number of roots per shoot. On propagation medium, root number and shoot number showed a slight positive correlation among clones, and both were positively correlated with the dry weight of shoots per shoot cluster and height of the shoot cluster (Table 5).

Root number per shoot cluster on propagation medium was positively correlated with the dry weight per shoot, among clones (CM) as well as within them (DCM) (Fig. 9; Table 6). There was no correlation between the dry weight per shoot and number of shoots per cluster among clones, but a negative one within clones (Fig. 10; Table 6).

As on propagation medium, the height of transferred shoots and root number on rooting medium were positively correlated among and within clones (Fig. 10; Table 6). Even though some clones never formed roots on the

Table 5. Correlations among clonal means of \ln (number of roots per shoot cluster + 1) (number of roots), \ln (number of shoots per shoot cluster) (number of shoots), total dry weight of shoots per shoot cluster (dry weight), and height of shoot cluster (height of cluster) of the two F_2 families a and b on propagation medium. (Brandt 1994)

Parameters (CM)	Family	Coefficient of correlation (R)	Significance (p)	Combined significance (p^*p)
No. of shoots versus number of roots	a ^a	0.189	0.424	0.0350
	b ^b	0.202	0.0825	
No. of shoots versus dry weight	a ^a	0.640	0.0024	<0.0001
	b ^b	0.480	0.0001	
No. of roots versus dry weight	a ^a	0.500	0.0248	<0.0001
	b ^b	0.586	0.0001	
No. of shoots versus height of cluster	a ^a	0.448	0.0478	<0.0001
	b ^b	0.438	0.0001	
No. of roots versus height of cluster	a ^a	0.530	0.0161	<0.0001
	b ^b	0.628	0.0001	

^a20 clones.

^b75 clones.

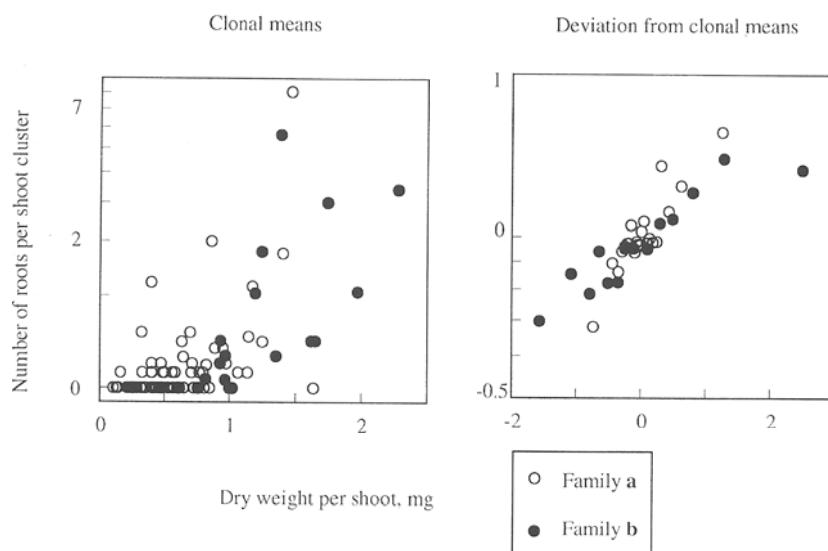


Fig. 8. Number of roots per shoot cluster as a function of dry weight per shoot, in two F_2 families a and b. Data for deviations from clonal means, DCM, are averages of 20 shoot clusters. For clonal means, CM, there were 14 shoot clusters per clone in family a and 6 in b. (Brandt 1994)

propagation medium and the shoots for the rooting experiment were taken only from a few subcultures, a slight correlation was found among clones for root formation on rooting medium with shoot size and root number on propagation medium (Table 6).

Table 6. Correlations among \ln (number of roots per shoot cluster +1) (number of roots), \ln (number of shoots per shoot cluster) (number of shoots) and dry weight per shoot (shoot dry weight) among and within clones of the two F_2 families a and b on propagation medium. Correlations between number of roots after 20 days on rooting medium (roots at 20 days) and height of shoots transferred to rooting medium (shoot height) among and within clones of family a. (Brandt 1994)

Parameters	Family	Coefficient of correlation (R)	Significance (p)	Combined significance (p^*p)
No. of shoots versus shoot dry weight within clones (DCM)	a ^a	-0.142	0.0229	<0.0001
	b ^b	-0.264	0.0001	
No. of shoots versus shoot dry weight among clones (CM)	a ^c	0.179	0.451	Not significant
	b ^d	-0.057	0.628	
No. of roots versus shoot dry weight within clones (DCM)	a ^a	0.283	0.0001	<0.0001
	b ^b	0.388	0.0001	
No. of roots versus shoot dry weight among clones (CM)	a ^c	0.687	0.0008	<0.0001
	b ^d	0.524	0.0001	
Roots at 20 days versus shoot height within clones (DCM)	a ^e	0.330	0.0001	
Roots at 20 days versus shoot height among clones (CM)	a ^c	0.794	0.0001	
Roots at 20 days versus height of shoot cluster on propagation medium among clones (CM)	a ^c	0.474	0.035	
Roots at 20 days versus root number on propagation medium among clones (CM)	a ^c	0.414	0.069	

^a274 shoot clusters.

^b447 shoot clusters.

^c20 clones.

^d75 clones.

^e754 shoots.

The correlations between number of roots and shoot size are significant for both clonal means (genetic factors) and deviation from clonal means (nongenetic factors). Therefore it seems that for *C. isophylla* the capacity for root formation in vitro depends simply on shoot size, whether a particular large or small size is caused by genetic or by other factors. These other factors, which occur randomly in this experiment, can be, e.g., differences in the shape and exact size of explants, and thus in the number of axillary buds present, and differences in temperature or irradiance at different locations within the growth chamber. On the other hand, the shoot number influences the shoot

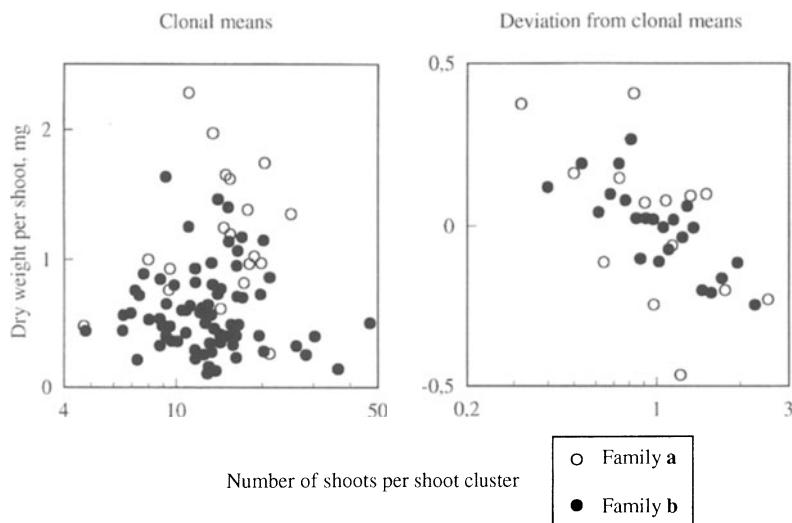


Fig. 9. Dry weight per shoot as a function of number of shoots per shoot cluster, in two F_2 families *a* and *b*. Data for clonal means, CM, are for 14 shoot clusters per clone in family *a* and 6 in *b*. Deviations from clonal means, DCM, are averages of 20 shoot clusters. (Brandt 1994)

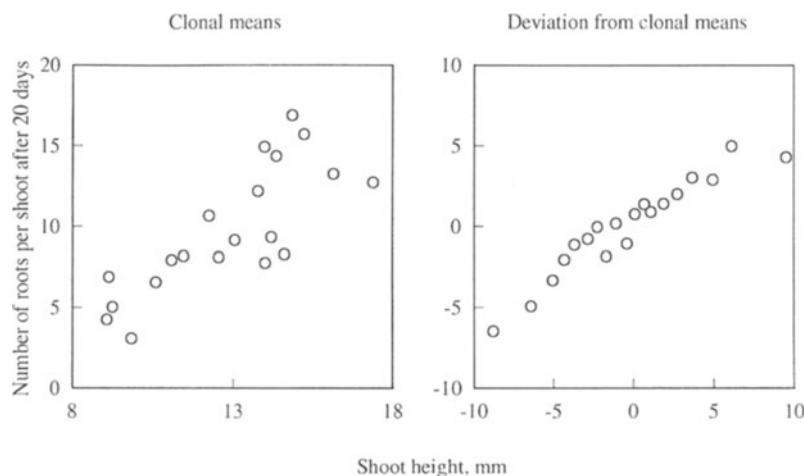


Fig. 10. Number of roots 20 days after placing excised shoots on rooting medium, as a function of shoot height at the time of excision. Data for clonal means, CM, are for 40 shoots per clone, deviations from clonal means, DCM, are averages of 40 shoots. Only data from family *a* were recorded. (Brandt 1994)

size only if it is determined by nongenetic factors, since the negative correlation is found only for deviations from clonal means, not for the clonal means. This means that refinements in culture conditions, leading to production of more shoots, are likely to lower the shoot size and thereby root formation.

However, increased shoot formation can be obtained by selection of clones with many large shoots without compromising root formation.

The lack of negative correlation between numbers of shoots and roots shows that effects that could possibly be caused by differences in the genetically determined auxin/cytokinin ratio are not an important source of interclonal variation in *C. isophylla*. Both number of shoots and number of roots are positively correlated with the total dry weight of shoots. Therefore, the growth rate, or general vigor of the shoot clusters, may be the most important difference between clones with high and low proliferation of shoots.

This study has confirmed the observations by Horn et al. (1988) that even small differences in the in vitro environment can promote the formation of alternatively many or large shoots, but it has also shown that selection of the best clones is a possibility for improving both simultaneously.

3 Summary and Conclusions

The genus *Campanula* comprises approx. 300 herbaceous, mostly blue-flowering species. Several are cultivated for cut flowers or pot plants. Micropropagation can be used for clonal propagation of a number of *Campanula* species. Propagation by basal parts of shoot clusters gives a reasonable multiplication rate without genetic aberrations, but is mostly of interest for propagation of disease-free stock plants, due to the relatively high cost of the plantlets. Initial explants are stem sections with one axillary bud, 2-mm sections are superior to smaller ones. If the buds are generative, they may develop into flowers in vitro. Using sections of the basal part of shoot clusters for propagation give better results than shoot tips or nodes. Propagating *C. isophylla* on MS with $4.4\mu\text{M}$ BA is superior to using media with less BA, and not significantly different from $8.8\mu\text{M}$ BA. Addition of NAA led to a greater number of roots, but also induced malformed roots and undesirable callus, so half-strength MS without growth regulators was preferred for root formation. In *C. carpatica* an acceleration of root formation was achieved using activated charcoal and 6% sucrose. Statistical analysis of the formation of roots and shoots in 95 genotypes showed that even small differences in the in vitro environment can promote the formation of alternatively many or large shoots, but also that selection of the best clones is a possibility for improving both simultaneously.

4 Protocol for *Campanula isophylla*

4.1 Explants

Preferably use vegetative shoots. Predisinfect in 1% Korsolin for 10 min, trim all cut surfaces, disinfect in 3% Korsolin for 5 min, rinse in sterile deionized water, and trim internodes and petioles further to an explant size of 2 mm, each explant with at least one bud. NaOCl with Tween may be used instead of Korsolin, using 0.3 and 1% for predisinfection and disinfection, respectively.

4.2 Propagation

Medium for propagation is MS with $4.4\mu\text{M}$ BA. Every 5 weeks apical parts of all elongated shoots are removed, leaving a 3-mm base. The basal parts of the shoot clusters are divided into sections, each with four 3-mm stem stubs.

4.3 Root Formation and Acclimation

Use well-sized shoots from the shoot clusters. Medium for root formation is MS with the concentration of macronutrients reduced to one-half, and no growth regulators. Transplant in a peat moss mixture that has been disinfested with heat (80°C overnight). Keep at high humidity for 10 days or until new leaves are in active growth.

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I.6 Micropropagation of *Coleus forskohlii* (Willd.) Briq.

K.P.S. CHANDEL and N. SHARMA¹

1 Introduction

1.1 Distribution and General Morphology

Coleus forskohlii (Willd.) Briq. (Syn. *C. barbatus* (Andr.) Benth. (Lamiaceae) is a herbaceous species of medicinal importance, found on dry hill slopes particularly in the subtropical, warm temperate climate zones in the Afro-Asian subcontinents. It has been recorded from mountainous regions of India, Nepal, Sri Lanka, Burma, Thailand, and parts of Africa. In India it grows wild in the Himalayan region, from the Shimla hills extending through the Kumaon and Garhwal hills, at an altitudinal range of 600–2300 m, in the Parasnath hills (Bihar) and in Gujarat and Western Ghats (Anonymous 1950; Husain et al. 1992).

The plant is herbaceous, attaining 30–60 cm in height, with perennial roots (Fig. 1D). The entire plant is aromatic. The roots are long, slender, often branched, and creamy-orange in color. The stem usually grows erect, and after attaining its maximum height (45–60 cm) it becomes decumbent when allowed to grow further. The inflorescence and flowers are typical of the family Labiatae. The roots, which constitute the economic product, are fasciculated, succulent, and tortuous or radially spread.

1.2 Economic Importance

Ayurvedic practitioners in India have used this plant to treat illness and various diseases, including cardiac troubles, since ancient times. The plant is now valued as a source for forskolin, an alkaloid used in the production of a drug for the treatment of glaucoma, congestive cardiomyopathy, and asthma, (de Souza et al. 1986; Valdes et al. 1987; Husain et al. 1992), owing to its adenylate cyclase stimulant activity. *C. forskohlii* is also valued for antiallergic activity (Gupta et al. 1991) and suppressing hair graying (Keikichi et al. 1988). The leaves are used as an expectorant and diuretic.

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Although diterpenoids are found in almost all parts of the plant, the roots are the main source (Shah et al. 1980), the major diterpenoid being forskolin (coleonol), upto 0.1% of the dry weight of the roots and 0.05% of the dry weight of the whole plant. The secondary metabolites are stored mainly in the cytoplasmic vesicles of cork cells in both fibrous and tuberous roots (Abraham et al. 1988).

Forskolin possesses positive inotropic and blood pressure-lowering activities through intravenous administration, is a CNS depressant, bronchodilator (Lichey et al. 1984), serves nerve regeneration, and lowers intraocular pressure (Caprioli and Sears 1983; Meyer et al. 1987). Besides forskolin, other diterpenoids present in the roots are the coleonols B, C, D, E, and F, coleosol, etc. (Husain et al. 1992).

1.3 Conventional Propagation and Need for In Vitro Culture

Due to limited cultivation of the plants, little documented information is available on cultivation practices. The plant is cultivated in Maharashtra and Gujarat in herbal/kitchen gardens. It can be propagated by seeds/stem cuttings. The best planting time in Maharashtra State is after the monsoon, and crop duration is 6 months.

Owing to its unique pharmacological properties and its potential as a blood pressure-lowering agent and in platelet aggregation, *C. forskohlii* has attracted worldwide attention in recent years. Due to the increasing requirements of the pharmaceutical industry, it became necessary to find an alternative plant source of forskolin. Thus, six species each of the genus *Coleus* and related taxa, *Plectranthus*, collected from different locations spread over the Indian subcontinent, were analyzed by de Souza (1991). However, the quantity of forskolin present in these plants was found to be very low, which made it difficult to produce an economically viable product.

Coleus forskohlii is the best-known source for the commercial production of forskolin. The pharmaceutical industry is largely dependent on wild populations for the supply of dried roots, estimated to be about 1000t. Further, limited efforts are being made to bring this species under cultivation. The most pragmatic approach to reducing the cost of the raw material would therefore be to select and cultivate high forskolin-yielding strains of *C. forskohlii* on a large scale. Vishwakarma et al. (1988) attempted to screen 38 genotypes collected from various locations to identify potential genotypes for forskolin. The genotypes exhibited a wide range for variation (0.01–0.44%); the km-2 line with 200.7 g/plant root dry matter and 0.40% forskolin was identified as a potential source for commercial cultivation.

The major hurdle faced at present is that the level of forskolin is very low and it seems difficult to produce economically. Moreover, the growth rhythm of the plant is comparatively slow and the alkaloid accumulation pattern is influenced by environmental and/or geographical conditions. In vitro techniques would enable stable production of forskolin.

2 In Vitro Propagation

2.1 Shoot Multiplication

A literature survey revealed only two reports of in vitro propagation of *Coleus forskohlii* (Sen and Sharma 1991; Sharma et al. 1991). Plants collected from natural habitats in the northern Himalayas and maintained ex situ were used

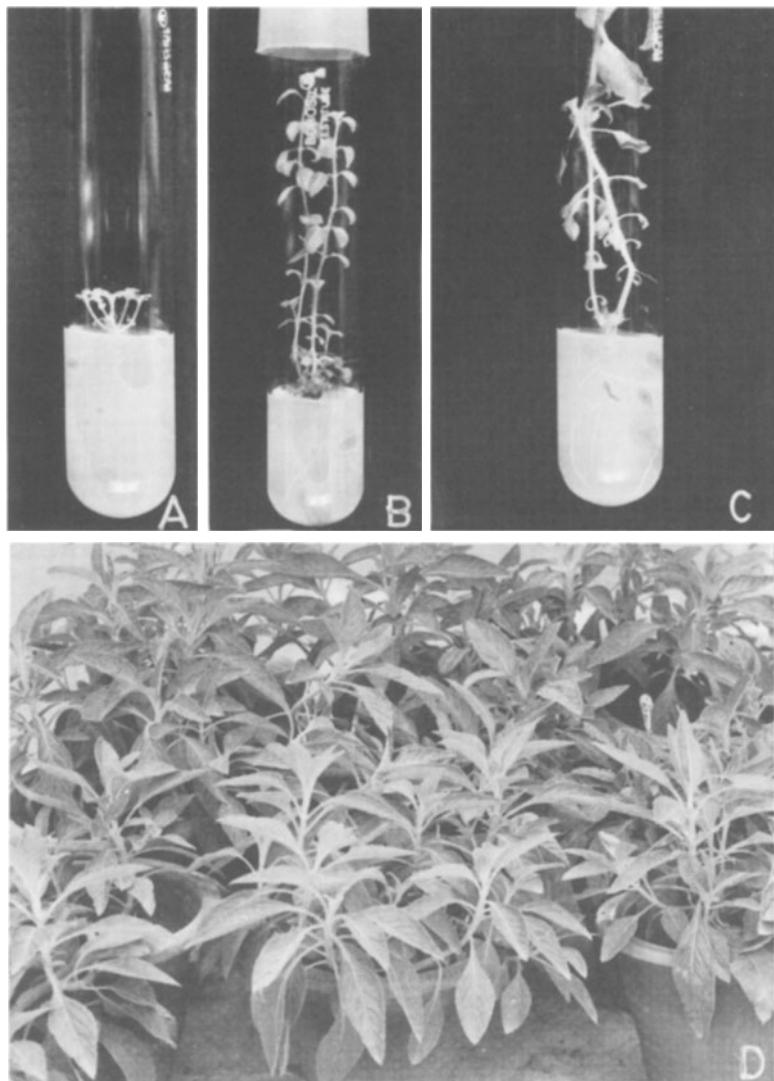


Fig. 1A–D. In vitro propagation of *Coleus forskohlii*. **A** Two-week-old culture of nodal segment showing multiple shoot formation. **B** Multiple shoots on MS + 2 mg/l kinetin + 1 mg/l IAA in 6 weeks. **C** Plantlet on MS + 1 mg/l IAA, after 4 weeks of culture. **D** Three-month-old plant established in earthen pots

as starting material (Sharma et al. 1991). Nodal stem segment (1–1.5 cm) from 6-month-old plants were used as explants. Stem segments were washed in teepol detergent for 15 min and later thoroughly washed under running tap water. Explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 min and implanted vertically onto MS medium (Murashige and Skoog 1962) supplemented with 2 mg/l kinetin and 1 mg/l IAA.

Single-node segments showed 50% bud break during the first passage on MS medium supplemented with 2 mg/l kinetin and 1 mg/l IAA. Upon transfer of these sprouted buds to fresh medium of the same composition, the shoots attained an average length of 4.3 cm with four to five nodes providing at least five cuttings per shoot (Fig. 1A,B). Following subculture of such individual cuttings, an average of 12 new shoots was produced every 6 weeks (Sharma et al. 1991).

In vitro multiplication of *C. forskohlii* was also simultaneously reported by Sen and Sharma (1991). Seedlings were raised aseptically from seeds of a high-yielding strain obtained from CIMAP, Lucknow. For shoot multiplication, 0.8–1-cm-long shoot tips with two cotyledonary leaves from 20- and 30-day-old seedlings and nodal segments, and also the excised leaves from 1-month-old regenerated shoots, were used as explants. Shoots multiplied within 20–25 days from shoot tip explants on MS supplemented with 2 mg/l BAP. The rate of multiplication was further enhanced by a gradual decrease in the level of BAP and its final omission after 4 months. Explants cultured on medium containing 2 mg/l BA, provided 150 shoots/shoot tips for the next multiplication cycle. The response varied with age of explants. However, shoot tips isolated from 30-day-old seedlings yielded the maximum number of shoots. Rate of shoot formation varied with the type of explants, the optimum response being obtained with shoot tips. Bud initials of the nodal segments led to the development of two axillary shoots, whereas the cut ends of leaves led to root formation only.

2.2 Rooting and Planting Out of In Vitro Plantlets

For rooting, individual shoots were placed on MS medium supplemented with various auxins. Maximum root induction was observed using IAA (1 mg/l), where 100% shoots rooted within 1 week of transfer and ca. 35 roots measuring 6 cm were produced in 4 weeks (Fig. 1C; Sharma et al. 1991). Rooted plantlets were transplanted to soilrite in small plastic pots and acclimatized at $25 \pm 3^\circ C$ with 16-h photoperiod for 3–4 weeks. They were initially supplied with half-strength MS solution for 1 week and subsequently with water. In vitro-produced plants were successfully established with almost 100% initial survival (Fig. 1D). These plantlets were then transplanted in earthen pots and kept outside. These in vitro raised plants grew well, flowered normally and raised three successive crops for three consecutive seasons. They exhibited no abnormality when compared to the original donor plant. These plants formed healthy tuberous roots (Fig. 2), which were tested for the presence of forskolin.

According to Sen and Sharma (1991), rooting occurred in 90% of the shoots on MS basal medium within 20 days. Rooted shoots were maintained



Fig. 2. Tubers of in vitro generated plants of *C. forskohlii*

on 50 ml of liquid half-strength MS basal medium, and then in sterilized water for 2–3 months; 60% survival of plantlets was achieved on transplanting. Chromosome count in root tips of in vitro generated plants showed the diploid nature ($2n = 28$).

2.3 Forskolin in In Vitro Raised Plants

One of the main objectives in developing micropropagation protocols is to raise material for a sufficient supply of raw material for commercial production. Thus, the estimation of forskolin content in in vitro generated material was an essential prerequisite. In vitro generated and field-grown plants were harvested after 6 months of growth, and the total produce of tuberous roots was shade-dried and powdered (Sharma et al. 1991). The forskolin content of these samples was estimated following the method suggested by Inamdar et al. (1984) and compared with the samples obtained from wild plants. The forskolin content estimated was about 0.1% on a dry weight basis, and it compared well with that reported earlier in wild plants (Shah et al. 1980). Thus, tubers from in vitro propagated plants were demonstrated to be a potential source of forskolin. Micropropagation of elite high-yielding selections, if linked with scientific cultivation on a large scale, could provide an effective alternative source of raw material for pharmaceutical industry.

The potential of shoot cultures and plantlets in vitro as a source of forskolin has been demonstrated (Sen and Sharma 1991; Sen et al. 1992). For

quantification of forskolin, 2-month-old swollen explants, showing shoot differentiation (after excision of emerging shoots) and 1-month-old rooted shoots were used.

The swollen differentiating mass growing on BAP contained 0.009% forskolin, whereas 1-month-old micropropagated plants revealed 0.01% forskolin on a dry weight basis in the whole plant before transfer to the pot, in comparison to 0.05% found in the whole mature *in vivo* plants. Interestingly, quantification of forskolin separately in the roots of these plants (which is the main site) could not be determined at this stage, because the roots were slender and very few in number. The 60-day-old swollen shoot differentiating mass without the emerging shoots was able to synthesize forskolin ($9-10 \times 10^{-3}\%$) normally found in the roots.

2.4 Callus/Cell Suspension Cultures and Forskolin

Callus cultures were initiated on MS + NAA (2 mg/l) + BAP (1 mg/l) using 6-8-mm-long hypocotyl segments from 30-day-old aseptically grown seedlings (Sen et al. 1992). Sixty-day-old cultures were subcultured and maintained in White's medium supplemented with NAA (1 mg/l) and BAP (1 mg/l). The initial callus was hard and green to white in color, becoming friable and yellowish to white when subcultured. After 60 days, small, white, ageotropic roots developed on the callus mass. This differentiated rhizogenic callus mass did not reveal the presence of forskolin. Thus, root differentiation in the callus mass was not related to the ability to produce forskolin, although the main site of synthesis or accumulation of forskolin is the roots of the mature plant. Mersinger et al. (1988) reported increased forskolin production along with visible differentiation. Cell cultures were initiated and maintained by subculturing every 4 weeks on B5 medium supplemented with 0.5 mg/l 2,4-D and 0.2 mg/l kinetin. The callus formed dark green, very hard and large aggregates with a diameter of 2 cm. Suspension cultures were established from this callus culture and cultivated on a medium containing 1 mg/l 2,4-D, 0.2 mg/l kinetin, 600 mg/l casein hydrolyzate and maintained either under continuous light or darkness on a rotary shaker (100 rpm, 24 °C), the subculture period was 12–15 days. No forskolin or other labdane diterpenoid was formed under these conditions.

To initiate the biosynthesis of secondary metabolites, the cell aggregates of one-and-a-half-year-old suspension cultures were transferred to an induction medium containing IBA (0.4–1 mg/l) instead of 2,4-D. During the first induction period of 14 days, only a very small amount of diterpenoids were formed. The largest amounts obtained were 314 mg/kg dry cell weight (with 0.4 mg/l IBA) and 25 mg/kg forskolin in the second and third induction period, respectively. Induction of forskolin biosynthesis occurred also on hormone-free medium. The addition of IBA effected a better response. The spectrum of diterpenoids in the cell cultures as analyzed by TLC and HPLC remained very similar to the root's diterpenoid spectrum in terms of both qualitative and quantitative attributes.

The major limitation of cell cultures is that suspension cultures gradually lose their capacity to form secondary metabolites during a cultivation period of 3–4 years. Thus, it becomes essential to establish new cultures. With these fresh cultures, optimum forskolin contents of over 1000 mg/kg dry cell weight in the second and third induction periods can be obtained (Mersinger et al. 1988). Maximum forskolin value achieved so far has been 1481 mg/kg dry cell weight in medium supplemented with glycine in place of casein for suspension cultures maintained under continuous light. In addition, amounts of 1,9-dideoxy forskolin (DDF) were also estimated; the concentration of forskolin was always found to be 1.6 to 2.5 times higher.

The scale-up of the procedure was performed in 20-l air lift bioreactors by Mersinger et al. (1988). Forskolin production increased to a maximum of 730 mg/kg after 19 days of fermentation. The concentration of DDF was 352 mg/kg. The cells increased from 2 g/l dry cell weight at the beginning to 9.2 g/l after 23 days of fermentation. The same group of workers was able to scale up the induction system in a 200-l pilot reactor. Their results indicated the feasibility of production of forskolin by a two-stage process at rates comparable to those of natural plants.

2.5 Root Culture

Root cultures offer an advantage over the conventional cell suspension culture and shoot cultures as they are more suitable for cultivation on a large scale (Mugnier 1988; Wilson et al. 1990). Moreover, many compounds that are not formed in undifferentiated cell suspension cultures can be produced in root cultures. Root cultures have been established using different culture systems. In *Coleus*, in vitro root cultures were initiated using 0.8–1-cm-long root tips from 3–4-cm-long in vitro propagated shoots (Sen et al. 1992). The roots grew and formed an entangled mass due to the formation of primary and secondary laterals on one-quarter MS medium with 0.5 mg/l IBA and 1% sucrose. The roots were cultured for 56 weeks and subcultured at 40-day intervals. The dried root mass after 40 days of growth produced forskolin in trace amounts. However, further standardization of experiments is essential for scaling up forskolin production.

Thirteen root cultures developed from primary callus initiated from internodal segments (strain W060) were established on B5 medium supplemented with 1 mg/l IBA and 600 mg/l casein hydrolyzate by Krombholz et al. (1992). The dry weight tripled after 21 days and the cultures consisted of long (6–20 mm), highly branched, glossy-looking roots, which tended to form densely packed root mass. At the end of each culture cycle, forskolin concentration of 127 to 1200 mg/kg on a dry weight basis was observed. The medium contained only traces of forskolin. The growth proceeded in almost linear fashion for more than 28 days and reached a stationary phase.

Detailed investigations on strain W060, which yielded on an average 770 mg forskolin/g dry mass, revealed that after transfer, the forskolin content

dropped to 200mg/kg and again started increasing after about 7 days. Root cultures were also established using cell suspension culture 11-DDF on medium containing 4.2mg IBA/l (Krombholz et al. 1992). Forskolin production ranged between 4 and 9mg/l forskolin/l due to variation in biomass production.

2.6 Transformed Root Culture

Successful attempts were made by Krombholz et al. (1992) to infect *C. forskohlii* with *Agrobacterium rhizogenes* to force the formation and subsequent in vitro culture of transformed roots. Transformed roots were established using surface sterilized young leaves. Transformation was confirmed by mannopine detection. All root cultures produced forskolin and its derivatives in amounts ranging from 500 to 1300mg/kg dry weight, corresponding to about 4–5kg/l. The transformed roots exhibited a productivity of 0.3mg forskolin/l/day, with a maximum of 4.5mg/l/day compared to 0.15mg/l/day in untransformed roots, maximal level of forskolin produced being 3.5mg/l.

The main feature of the system was that the root cultures retained their ability to produce the diterpenes over a 2-year period. Scale-ups of the cultivation procedures were also performed by the same group of workers in 20-l glass jars with a working volume of 10–13l. Forskolin production in the bioreactor was better than in shake flasks, where a level of 14mg/l could be achieved after 21 days.

3 In Vitro Conservation

C. forskohlii is becoming increasingly endangered, and if the collection of the natural populations is allowed to continue on the present scale, this species may become extinct. It is already listed as a vulnerable species (Gupta 1988). Recently, a total synthesis of (\pm)-forskolin was reported (Hashimoto et al. 1988; Corey et al. 1988), which may divert the attention of the researcher for a short while and lead to saving this valuable species as a natural product source. However, in view of the dwindling population, it has become imperative to conserve this plant species by using in vitro technology.

The in vitro strategy employed at NFPTCR aimed at reducing the growth rate of cultures to avoid too frequent sub-culturing. The experiments devised to standardize short/medium-term in vitro conservation involved low temperature incubation (5, 10, 15°C), media modification, or a combination of both. The results obtained indicate that shoot cultures can be conserved in vitro for more than 12 months on shoot culture medium at normal culture room conditions (+25°C) (Sharma et al. 1995). Low-temperature incubation and media modifications in terms of inclusion of mannitol (to 4%) or increasing sucrose

were not found beneficial in enhancing the shelf life of in vitro cultures of *C. forskohlii* (Sharma and Chandel, unpubl.). Based on experimentation, storage in simple medium at 25°C appears to be the most desirable method, as it does not subject the cultures to any kind of stress of growth inhibitory treatment. In vitro cultures of *Coleus* produce roots by about 3 months. Rooted shoot cultures have the added advantage in conservation of being able to absorb nutrients from the medium, especially at later stages when water and nutrients are nearly exhausted, compared to the shoot cultures without roots.

4 Conclusion

In vitro propagation, although efficient, is still limited due to the high cost. Encapsulation of explants for direct germination under controlled greenhouse conditions can be of considerable advantage and potential in scaling up the production. Callus and cell suspension cultures can also serve for pharmaceutical production of forskolin. A two-step process has already been reported for scaling up the production of forskolin (Mersinger et al. 1988).

Although the establishment of callus cultures and studies related to forskolin production have been reported, the regeneration of plantlets from callus has still to be achieved. Root cultures (transformed and untransformed) are also a potential source of forskolin at a commercial level. Until a viable alternative system is established, the natural reserves remain the sole source of exploitation. Thus collection of germplasm, selection of high-yielding strains; and their in vitro conservation are strongly advocated.

5 Protocol

5.1 Establishment of Cultures

To initiate cultures, nodal stem segment (1–1.5 cm) from 6-month-old plants are used as explants. Stem segments are washed in teepol detergent for 15 min and later thoroughly washed under running tap water. The explants are surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 min and implanted vertically onto MS medium (Murashige and Skoog 1962) supplemented with 2 mg/l kinetin and 1 mg/l IAA.

5.2 Shoot Multiplication

After 4–6 weeks, the shoots are subcultured onto fresh medium of the same composition. Individual node segments are excised and implanted vertically onto semisolid nutrient medium. Within 2–6 weeks each explant develops new shoots. This shoot multiplication cycle can be repeated as required. When an adequate number of shoots has been produced, some material is kept for further shoot multiplication and some is transferred for rooting.

5.3 Rooting

For rooting, individual shoots with two to three nodes from in vitro multiplied cultures are used. Remove basal leaves and trim stem base and inoculate onto MS medium containing 1 mg/l IAA.

5.4 Planting Out

For field transfer and establishment, 6- to 8-week-old plantlets with well-developed roots were removed from the culture tubes, washed free of agar, and transplanted in a sterilized mixture of garden soil and sand (1:1) or soilrite. They were acclimatized for 3–4 weeks at 25 °C.

5.5 Culture Medium and Culture Conditions

The basal medium used was MS (Murashige and Skoog 1962) containing 0.8% agar and 3% sucrose. This was supplemented with kinetin or BAP alone or in combination with IAA. The pH of the medium was adjusted to 5.8 prior to autoclaving at 1.08 kg/cm² (121 °C) for 15 min. Cultures were grown in test tubes (150 mm × 25 mm) containing 20 ml of medium, and cultures were maintained at 25 ± 3 °C with 16-h photoperiod (2800–3000 lx) provided by cool, white fluorescent tubes. Each experiment was replicated 24 times and all experiments were repeated twice, and subcultured every 4–6 weeks.

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I.7 Micropropagation of *Ctenanthe lubbersiana* Eichl. and *Maranta leuconeura* Morren var. *Tricolor* (Marantaceae)

F. SCARAMUZZI and G. APOLLONIO¹

1 Introduction

Members of the family Marantaceae are ornamental tropical perennial foliage plants generally provided with rhizomes. This family of these monocotyledons includes 27 genera and about 300 species, whose original areas are the tropical zones of South America. The leaves are basal, double, or triple and emerge from an articulate petiole. The flowers are insignificant, hermaphrodite, irregular, provided with a calyx, a corolla, and five stamens, of which only half are fertile. The ovary is 1–3-locular with a single ovule in each cavity; the fruit is a berry or a nut.

Economically, Marantaceae are an important source of food. *Maranta arundinacea* L. of tropical America, also cultivated in the tropical regions of the West Indies and Africa as an industrial plant, produces “the arrowroot of the West Indies and Antilles”. Its rhizomes consist of about 27% of a potato flour, which is prepared by crushing and washing to free the starch. The starch granules resemble those of potatoes, but are much smaller and pear-shaped.

Arrowroot is important both for the feeding of indigenous populations and for special diets requiring a readily digestible starch (Tonzig 1982).

Other Marantaceae have economic value as highly esteemed ornamental plants (*Calathea*, *Thalia*, *Stromanthe*, *Maranta*, etc.).

The above-mentioned plants are often cultivated in gardens or greenhouses for their ornamental foliage. Such species multiply asexually from plant division or from tip cuttings. Multiplication by seeds is rare, because they rarely germinate. The Marantaceae are attacked by a number of pests and pathogens. Diseases are produced by fungi, viruses, and bacteria.

In *Maranta* spp. fungal diseases are caused essentially by *Rhizoctonia solani* (stem rot), *Glomerella cincta* and *Phyllosticta marantae* (foliar mottles) (Tesi 1985; Zechini D'Aurelio et al. 1992). In *Calathea*, *Maranta*, *Ctenanthe*, and *Stromanthe*, *Drechslera setariae* causes leaf lesions (Simone and Brunk 1983). Viral diseases in *Maranta leuconeura kerkoveana*, showing foliar chlorotic spots and ringspots, are essentially caused by CMV (cucumber mosaic virus; Hearon 1979). Issa (1963) reports *Murcha bacteriana* (= bacterial wilt), a new disease of arrowroot (*Maranta arundinacea*).

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2 In Vitro Culture Studies

The in vitro work done so far concerns only *Maranta leuconeura* (Henny 1980). This author reported that this ornamental tropical foliage plant is normally propagated asexually from tip cuttings. Although seeds can be produced by hand pollination, they rarely germinate. The author describes the use of embryo culture to increase seed germination, the results are summarized in Tables 1 and 2.

3 Micropagation

Two species are examined: *Ctenanthe lubbersiana* Eichl. (= *Phrynum lubbersi* Hort. = *Stromante lubbersiana* Morren) and *Maranta leuconeura* Morren var *tricolor* (= *M. messangeana* Morren = *Calathea messangeana* Hort. = *Calathea kerchoveana* Hort.). Both species are originally from Brazil and are perennial herbaceous plants provided with rhizomes.

3.1 Sterilization and Conditions of Explant Culture

For both species, the nodal meristems (4–5 mm) were isolated from stems several times in the year (Scaramuzzi et al., 1992), owing to possible variations (Gautheret 1959), and were sterilized with a 1.2% Cl_2 in a solution of sodium hypochlorite for 15 min. They were then washed three times with sterile distilled water before inoculation. The cultures were maintained at $25 \pm 1^\circ\text{C}$ with a photoperiod of 14-h light (1200 lx).

Murashige and Skoog's medium (1962) (MS) contained 3–3.5% sucrose, 1% agar, organic substances, and the following combinations of auxins and cytokinins were employed:

- | | | |
|-----------------------------------|----------------|----------------------|
| a) MS basal medium | Control | |
| b) MS + IAA 1–5 mg/l + kin 1 mg/l | } | Multiplication media |
| c) MS + IAA 1–5 mg/l + BAP 5 mg/l | | |
| d) MS + NAA 1 mg/l + kin 1 mg/l | | |
| e) MS + NAA 1 mg/l + BAP 5 mg/l | | |
| f) MS + IBA 10 mg/l | Rooting medium | |

Table 1. Germination and callus production of *Maranta leuconeura* embryos after 10 weeks on 4 different tissue culture media (20 cultures per treatment). (Henny 1980)

Medium	Germination (%)	Callus rating
Emsweller et al.	35	1.6
Schenk-Hildebrandt	50	2.0
Linsmaier-Skoog	65	4.0
Norstog	30	4.7

Table 2. Germination and callus production of *Maranta leuconeura* embryos as affected by 2iP in a Linsmaier and Skoog medium (20 cultures per treatment). (Henny 1980)

2iP (mg/l)	Germination (%)					Callus rating
	Time in culture (weeks)					
	2	4	6	8	10	
0	0	25	50	80	85	3.7
0.1	0	35	45	50	65	3.1
1	0	50	70	75	80	3.1
10	10	75	80	95	95	2.4
30	30	100	100	100	100	1.7

The medium MS + IAA + 2iP was less favorable to growth than other media and was not utilized further.

The pH of the solution was adjusted to 5.8 and the medium sterilized at 110°C for 20 min.

3.2 Meristem Cultures

3.2.1 *Ctenanthe lubbersiana* Eichl.

The growth of nodal meristems is evident after 6–7 weeks of culture. The utilization of these explants permits a good percentage of success also in subcultures. The nodes contain in general three meristems (Fig. 1), but produce single shoots (at times double or triple) by abortion of the others (Figs. 2, 3, 4), and no callus, independently of hormonal contents. Roots (R_1) are produced on the same medium after shoot development. Transfer to MS + IBA medium, without cytokinins, further increases the growth of shoots and roots.

The result is the same on all media utilized and previously indicated, except on MS basal medium, on which growth is weak and slow.

In old cultures (11–12 months) rhizomes are produced in vitro: they are well developed and provided with visible circular sheaths (Fig. 5).

It is possible to practice subcultures by using both segments of newly formed rhizomes and nodal meristems of the previous generation (Fig. 6).

Culture of the latter, which produces a single shoot, is not favorable for productivity so that it is necessary to utilize segments of newly formed rhizomes, that each produce a single shoot.

The behavior of subcultures is similar to that of initial cultures as regards hormone combinations. It is possible to have three or more morphologically similar generations in succession (Figs. 7, 8). The plantlets obtained after 3–4 months are ready to be transferred to the soil, where they easily continue to grow (Fig. 9).

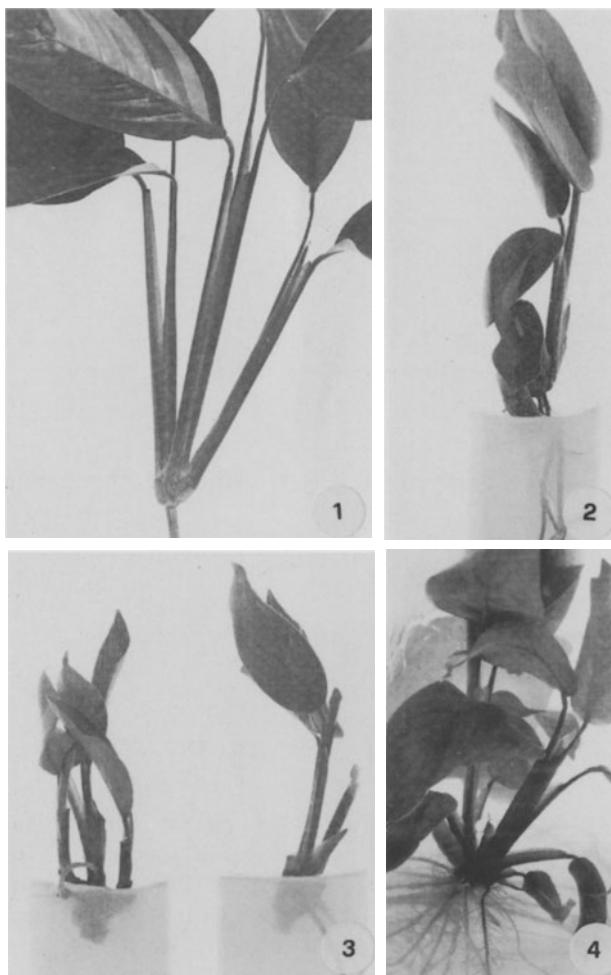


Fig. 1. Detail of a triple node with foliar bases from which initial nodal explants are isolated
Fig. 2. Single shoot with roots and no callus, cultivated on medium (b) = MS + IAA + kin; it derives from the central meristem of a node

Fig. 3. On the *left* a rare triple shoot; on the *right* a single shoot with roots and no callus, derived from a lateral meristem after abortion of the others

Fig. 4. Double-rooted plantlet derived from a triple node cultivated on medium (d) = MS + NAA + kin

3.2.2 *Maranta leuconeura* Morren var. *tricolor*

This species is herbaceous, provided with several roots and a few long thin rhizomes. The explants consist of nodal meristems (Fig. 10) easy to sterilize. The utilization of these cultures permits 80–90% success.

Growth is evident after 4–5 weeks of culture: single shoots and no callus are produced on all media after 30–40 days.

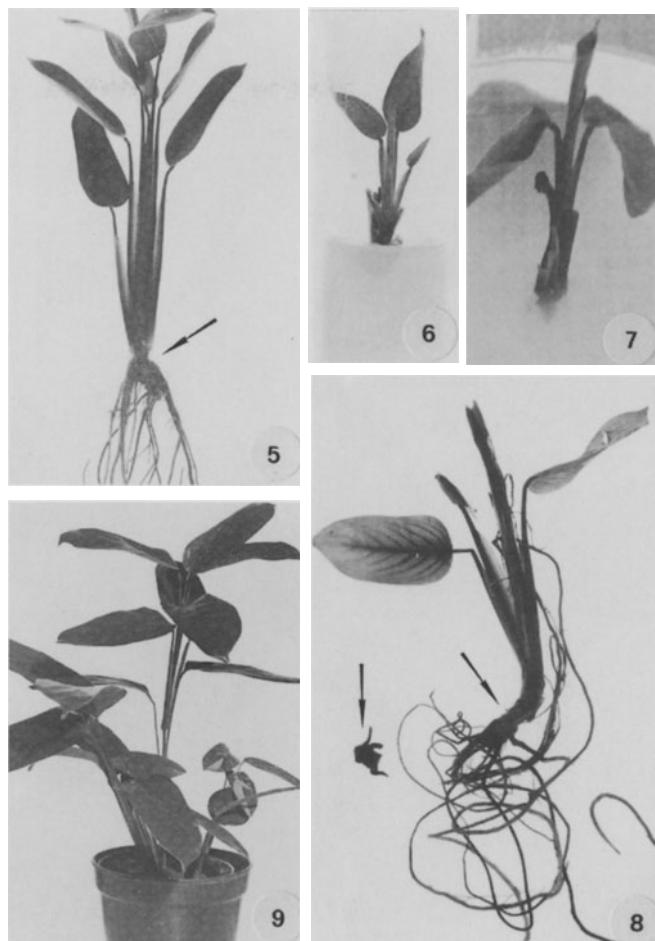


Fig. 5. First generation plantlet at advanced culture stage (about 14 months) that has produced a rhizome (arrow), whose segments will be utilized for subcultures

Fig. 6. Second generation shoot obtained from a nodal meristem of a first-generation plantlet

Fig. 7. Third generation shoot derived from a disk of a second-generation rhizome

Fig. 8. Second generation plantlet which shows a newly formed rhizome and a small disk (indicated by two arrows)

Fig. 9. Plantlet in pot grown in culture (4 months old)

Roots (R_1) are produced on the same medium, after shoot development, only on medium (d) (Fig. 11a) and on the basal medium. On the other media, (b), (c), and (e), the results are similar, i.e., production of single shoots and no callus, but absence of roots.

It is necessary to transfer to MS + IBA (rooting medium) to promote root growth (R_2). At an advanced culture stage, the shoots produce long, thin rhizomes (Fig. 11b), which are prolific if they are bound to the previous plant, and produce successive shoots (two to three or more) with greater facility than

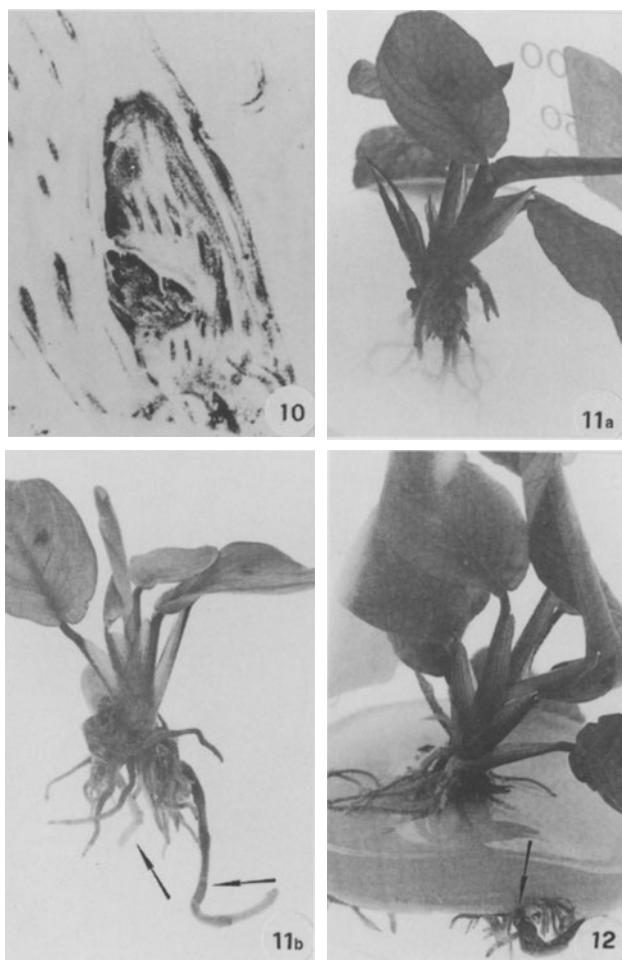


Fig. 10. Longitudinal section of a nodal meristem with foliar bases

Fig. 11. a First generation plantlet derived from a nodal meristem cultivated on medium (d) = MS + NAA + kin

Fig. 11. b Rooted shoot with two rhizomes (indicated by *two arrows*) with circular sheaths and no callus

Fig. 12. Complete plantlet with roots and rhizomes, one of which has produced a young shoot (*arrow*)

in situ (Figs. 12,13). Positive results are possible by utilizing several plantlets bound together or in succession, all provided with roots.

New plantlets are produced even if a well-developed shoot is isolated and subcultured. In contrast, segments of rhizomes and young isolated shoots are inactive.

The production of successive shoots, to the fourth or fifth generation, (Fig. 14) made it possible to compensate for the production of initial single shoots and thus make *in vitro* culture of the species advantageous.

The plants obtained are ready to be transferred to the soil (Fig. 15), where they continue to grow easily.

In conclusion, *Ctenanthe lubbersiana* and *Maranta leuconeura* var. *tricolor*, species that grow slowly in vivo, react more actively in vitro.

In initial cultures and subcultures, the production of single shoots, absence of callus, and consequent lack of differentiation of adventitious buds made favorable results impossible.

It is possible, however, to obtain subcultures by utilizing fragments of rhizomes in *Ctenanthe* and secondary shoots from prolific rhizomes in



Fig. 13. First generation plantlet with a rhizome which has produced a second-generation shoot. The latter, in turn, produces a rhizome with a third-generation shoot (indicated by two arrows). The complete culture is about 5 months old

Fig. 14. Third generation plantlet which has produced four of fourth-generation shoots from different rhizomes

Fig. 15. Plantlet in pot 3 months after culture

Table 3. Summary of results obtained in culture of *Ctenanthe lubbersiana* explants with different hormone substances (40 cultures per treatment)

Hormone combination (mg/l)	Initial cultures				Subcultures							
	Morphogenetic response of nodal meristems % = 20-30				Morphogenetic response of rhizome segments % = 30-40				nodal meristems % = 30-40			
	S	C	R ₁	R ₂	S	C	R ₁	R ₂	S	C	R ₁	R ₂
a) MS basal medium	+	-	+									
b) MS + IAA = 5 + kin = 1	+	-	++		+	-	+		+	-	+	
c) MS + IAA = 5 + BAP = 5	+	-	++		+	-	+		+	-	+	
d) MS + NAA = 1 + kin = 1	+	-	++		+	-	+		+	-	+	
e) MS + NAA = 1 + BAP = 5	+	-	++		+	-	+		+	-	+	
f) MS + IBA = 10				++				++				++

MS = Murashige and Skoog medium; IAA = indol-acetic acid; NAA = naphthalene-acetic acid; IBA = indol-butyric acid; kin = 6-furfuryl-amino-purine; BAP = benzyl-amino-purine; S = shoot; C = callus; R₁ = roots on the same medium; R₂ = further growth of roots on rooting medium; % = percentage of productivity explants; ++ = full activity; + = average activity; - = no activity.

Table 4. Summary of results obtained in culture of *Maranta leuconeura* var. *tricolor* explants with different hormone substances (40 cultures per treatment)

Hormone combination (mg/l)	Initial cultures				Subcultures							
	Morphogenetic response of nodal meristems % = 80-90				secondary shoots % = 80-90				nodal meristems % = 10			
	S	C	R ₁	R ₂	S	C	R ₁	S	C	R ₁		
a) MS basal medium	+	-	+		+	-	+	+	-	+	+	+
b) MS + IAA = 5 + kin = 1	+	-		+	+	-	+	+	-	+	+	+
c) MS + IAA = 5 + BAP = 5	+	-		+	+	-	+	+	-	+	+	+
d) MS + NAA = 1 + Kin = 1	+	-	+		+	-	+	+	-	+	+	+
e) MS + NAA = 1 + BAP = 5	+	-		+	+	-	+	+	-	+	+	+
f) MS + IBA = 10				++								

Maranta. The average behavior of cultures and subcultures is shown in Tables 3 and 4 and in Figs. 16 and 17 respectively, for *Ctenanthe* and *Maranta*.

4 Summary and Conclusions

The results obtained demonstrate that different explants of *Ctenanthe lubbersiana* Eichl. and *Maranta leuconeura* Morren var. *tricolor* (two Marantaceae species with variegated ornamental leaves) showed organogenetic potential on the basal medium.

a) Initial cultures

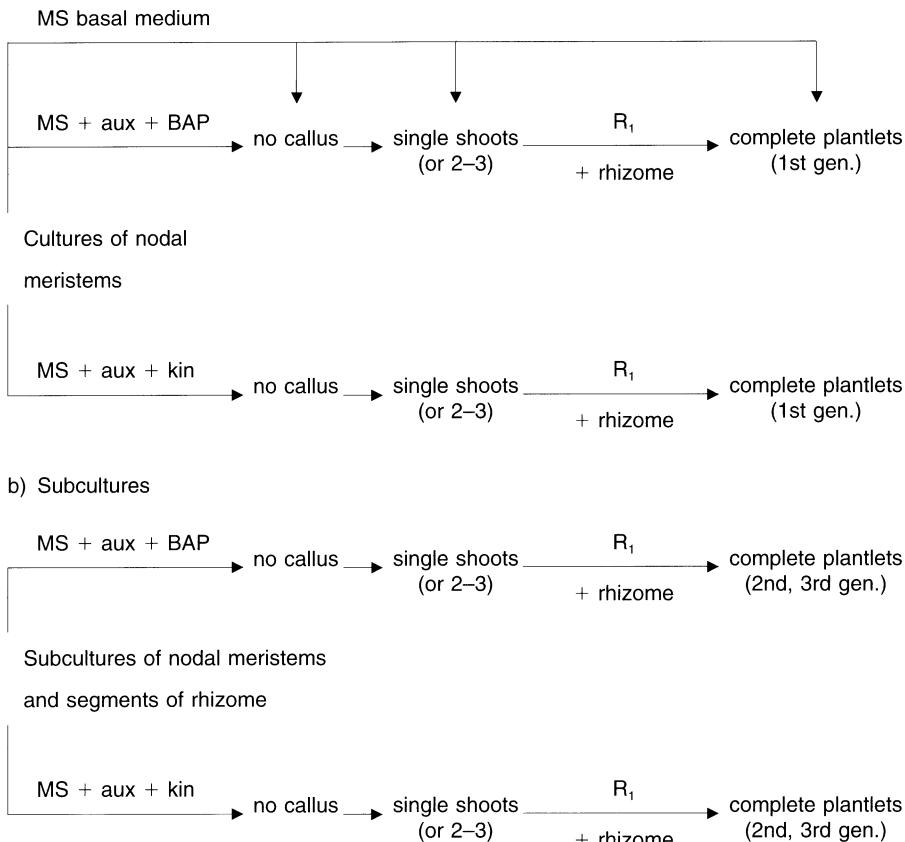


Fig. 16. Behavior of initial cultures and subcultures of *Ctenanthe lubbersiana*. R_1 , roots on the same medium; aux, IAA or NAA; MS, Murashige and Skoog medium; BAP, benzyl-amino-purine; kin, furfuryl-amino-purine

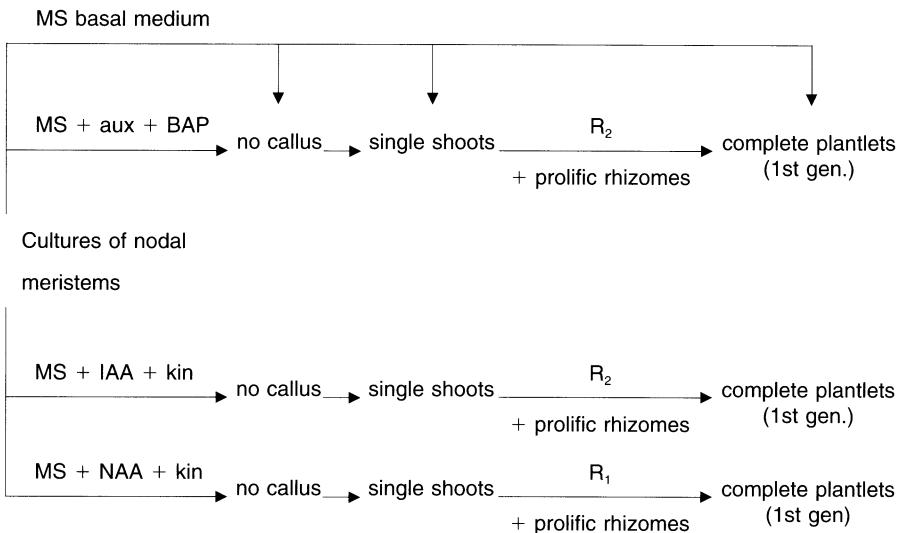
Association of growth regulators increased the activity of explants and root formation. For initial cultures of *Ctenanthe*, it was possible to successfully use nodal meristems that produced single (double or triple) shoots and no callus, independently of hormonal contents. In later culture rhizomes are produced.

Subcultures are possible by using segments of newly formed rhizomes and nodal meristems of the previous generation.

In *Maranta*, it was also possible to use nodal meristems that produced single shoots and no callus, independently of hormonal contents. Many roots are produced and, in later culture, some long thin rhizomes. These rhizomes are prolific if they are bound to the initial plant, and they produce successive plantlets more easily than in situ.

It was possible to have three or more generations in succession. New plantlets are produced even if an already well-developed shoot is isolated and

a) Initial cultures



b) Subcultures

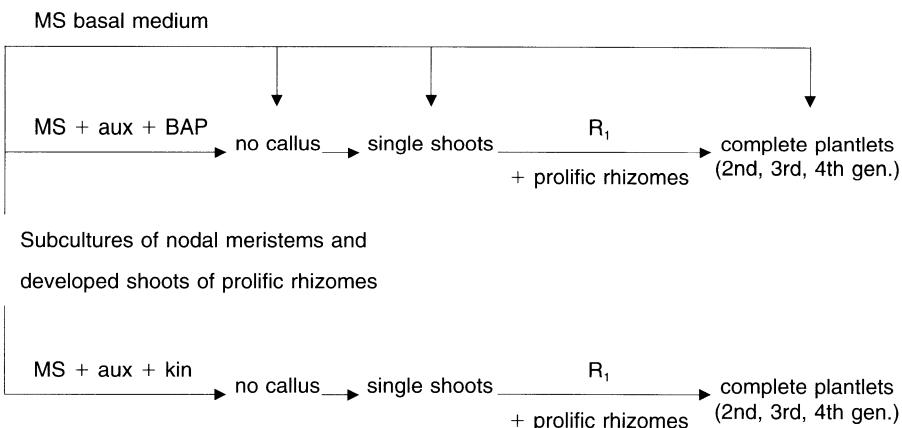


Fig. 17. Behavior of initial cultures and subcultures of *Maranta leuconeura* var. *tricolor*. R_1 , roots on the same medium; R_2 , roots on the rooting medium; aux, IAA or NAA; MS, Murashige and Skoog medium; BAP, benzyl-amino-purine; kin, furfuryl-amino-purine

subcultured. In contrast, segments of rhizomes and young isolated shoots are inactive.

In short, for both the species considered the advantage of in vitro culture is possible by using newly formed rhizomes, which compensates for the lack of multiplication in initial single shoots.

The commercial aspects of the culture of Marantaceae are limited to the genus *Calathea*. The number of plants cloned in vitro in The Netherlands was: in 1985, 240; in 1986, 10 000 (Pierik 1987).

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I.8 Micropropagation of *Cyclamen persicum* Mill.

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1 Introduction

The genus *Cyclamen* (family Primulaceae) contains 19 species (Ishizaka and Uematsu 1992), which are distributed in and near the Mediterranean region. Plants of this genus have a tuber and elegant nodding flowers. To date, only *C. persicum* has been used as a commercial plant. Although *C. persicum* is a diploid with $2n = 48$ chromosomes originally, breeding was formerly carried out at the tetraploid level. Legro (1959) reported diploids with $2n = 48$ chromosomes, tetraploids with $2n = 96$ chromosomes, and tetraploid aneuploids with $2n = 90, 92, 94$, and 95 chromosomes in cyclamen cultivars. Nevertheless, the number of diploid cultivars has been increasing since Wellensiek (1961) introduced breeding at the diploid level. As tetraploid cyclamen breeding is difficult, his discovery of a diploid cyclamen, i.e., diploid with large flowers was promising. At present, there are diploid and tetraploid cultivars, and desirable characteristics are observed in each ploidy, for example, the yellow-flowered cyclamen (Miyajima et al. 1991) in the diploid cultivars.

Cyclamen is commercially propagated by seeds. In fact, vegetative propagation is desirable for the propagation of cyclamen because there is some variation in seed-derived cultivars and because F_1 seeds, the breeding of which has been advanced more and more successfully, are relatively expensive (Wainwright and Harwood 1985). Nevertheless, the daughter tuber is not formed and division and splitting are difficult in cyclamen. Although Mütze (1927) and Nakayama (1979, 1980) reported that adventitious shoots were induced by removing the shoot and notching the tuber *in vivo*, it is difficult to regard these as economical methods. Mass propagation by tissue culture is therefore expected to be the effective propagation method in cyclamen.

Since 1989 we have been working on in vitro micropropagation, especially organogenesis and embryogenesis, for mass propagation and breeding of cyclamen. This chapter reviews mass micropropagation and micropropagation in the breeding of cyclamen.

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2 Brief Review of Micropagation of Cyclamen

Mayer (1956) made the first report on in vitro regeneration via organogenesis from the tuber segments of cyclamen. From that time until the 1970s, many reports were made (Stichel 1959; Loewenberg 1969; Okumoto and Takabayashi 1969; Geier 1977). Tuber tissues were also used as explants for organogenesis in these reports. Micropagation from cyclamen tuber tissue was, however, very difficult without the use of antibiotics because tuber tissues contained systemic microorganisms, especially bacteria. Moreover, antibiotic substances generally obstruct organogenesis in cyclamen.

It has been reported that anther (Geier et al. 1979; Miura 1988), etiolated petiole (Ando and Murasaki 1983; Murasaki and Tsurushima 1988), and aseptic seedling tissues (Wainwright and Harwood 1985; Hawkes and Wainwright 1987; Oohashi et al. 1989; Takamura et al. 1993) could be used as explants without microorganisms. These tissues regenerate plantlets via organogenesis.

Although all the above reports are on plant regeneration via organogenesis, some reports on somatic embryogenesis are also available in cyclamen (Fersing et al. 1982; Wicart et al. 1984; Otani and Shimada 1991; Kiviharju et al. 1992; Oohashi et al. 1992; Takamura et al. 1995). The tissues used as explants were leaf (Wicart et al. 1984; Otani and Shimada 1991), petiole (Wicart et al. 1984), ovary (Wicart et al. 1984; Kiviharju et al. 1992), anther (Kiviharju et al. 1992), roots (Oohashi et al. 1992), and aseptic seedling tissues (Takamura et al. 1995). In general, somatic embryogenesis is one of the suitable methods for micropagation, since embryogenic culture optimally can produce a large number of embryos per culture flask, many more than the multiple shoots generated adventitiously via organogenesis.

3 Plant Regeneration Via Somatic Embryogenesis from Aseptic Seedling Tissue

Aseptic seedlings were used for embryogenesis, since the tissues had no microorganisms and it was considered that their explants had higher potential for plant regeneration than tissues of matured plants.

To obtain aseptic seedlings, dry seeds were soaked in sodium hypochlorite solution (about 2% available chlorine) containing a few drops of detergent for 10 min, and rinsed three times with sterile distilled water. The disinfected seeds were sown on one-third MS (Murashige and Skoog 1962) medium (3% sucrose, 0.3% gellan gum), then incubated at 20°C under dark conditions for 3 weeks followed by 16-h day length (daylight, about $30\mu\text{mol/m}^2/\text{s}$).

Seven-week-old aseptic seedlings were divided into cotyledons, petioles, tubers, and roots. The cotyledons, petioles, and tubers were divided into five, four, and eight segments, respectively, and the roots were cut into about 5 mm length and used as explants.

3.1 Effects of Media Composition and Incubation Condition on Embryogenesis

In all experiments, inorganic and organic nutrients of MS solidified with 0.2% gellan gum were used as basic media.

3.1.1 Effects of Plant Growth Regulators and Sucrose on Embryogenesis

To investigate the effects of plant growth regulators and sucrose on embryogenesis, 2,4-D at 0.5, and 50 μ M and kinetin at 0, 0.5, 5, and 50 μ M were tested in each basic medium with 6% sucrose. Sucrose concentrations ranging from 0–9% were tested in medium containing 5 μ M 2,4-D and 0.5 μ M kinetin. Cultures were maintained at 25 °C in the dark. The number of explants forming callus and embryoid were recorded after 6 weeks of culture. The calli produced were transferred to MS medium without plant growth regulator. After 5 weeks of subculture, the number of calli forming embryoids and number of embryoids formed were recorded. In these experiments, explants from aseptic seedlings of Anneke were used.

The effects of plant growth regulators on embryogenesis are summarized in Fig. 1. Callus formation and embryoid formation were observed in all four types of explants, namely, cotyledon, petiole, tuber, and root, from aseptic seedlings. Earlier, Wainwright and Harwood (1985) had not been able to induce somatic embryos from the aseptic seedling tissues of Rosamunde, and Oohashi et al. (1992) reported that somatic embryos were formed only from the root explants of the young seedlings via organogenesis of Chopin.

All types of explants showed a much higher number of embryoids and a greater percentage of explants forming embryoids on the medium with 2,4-D and kinetin at 10:1 concentration ratio. The best response appeared to be at 5.0 μ M 2,4-D with 0.5 μ M kinetin. Kiviharju et al. (1992) reported that a higher percentage of somatic embryo production was observed in the medium with 2,4-D and coconut milk than without coconut milk; Otani and Shimada (1991) also reported that kinetin at a low concentration (0.1 mg/l) stimulated embryogenic callus formation in the medium with 1 mg/l 2,4-D. These findings suggest that the presence of auxin (2,4-D) and cytokinin-like substance in the medium, and the relative ratio of auxin to cytokinin were important factors for the embryogenesis of cyclamen.

Embryogenesis was observed in primary culture on the medium with 5 μ M 2,4-D, and 0.5 μ M kinetin, although no embryoid was produced in primary culture on the medium with 50 μ M 2,4-D and 5 μ M kinetin. This indicates that embryogenesis was inhibited or delayed by a high concentration of plant growth regulators, perhaps especially auxin. Optimal concentrations of plant growth regulators in the medium varied according to explant types. Tuber explants showed the highest embryoid number in the medium with 50 μ M 2,4-D and 5 μ M kinetin, while the medium with 5 μ M 2,4-D and 0.5 μ M kinetin was optimal for root explants.

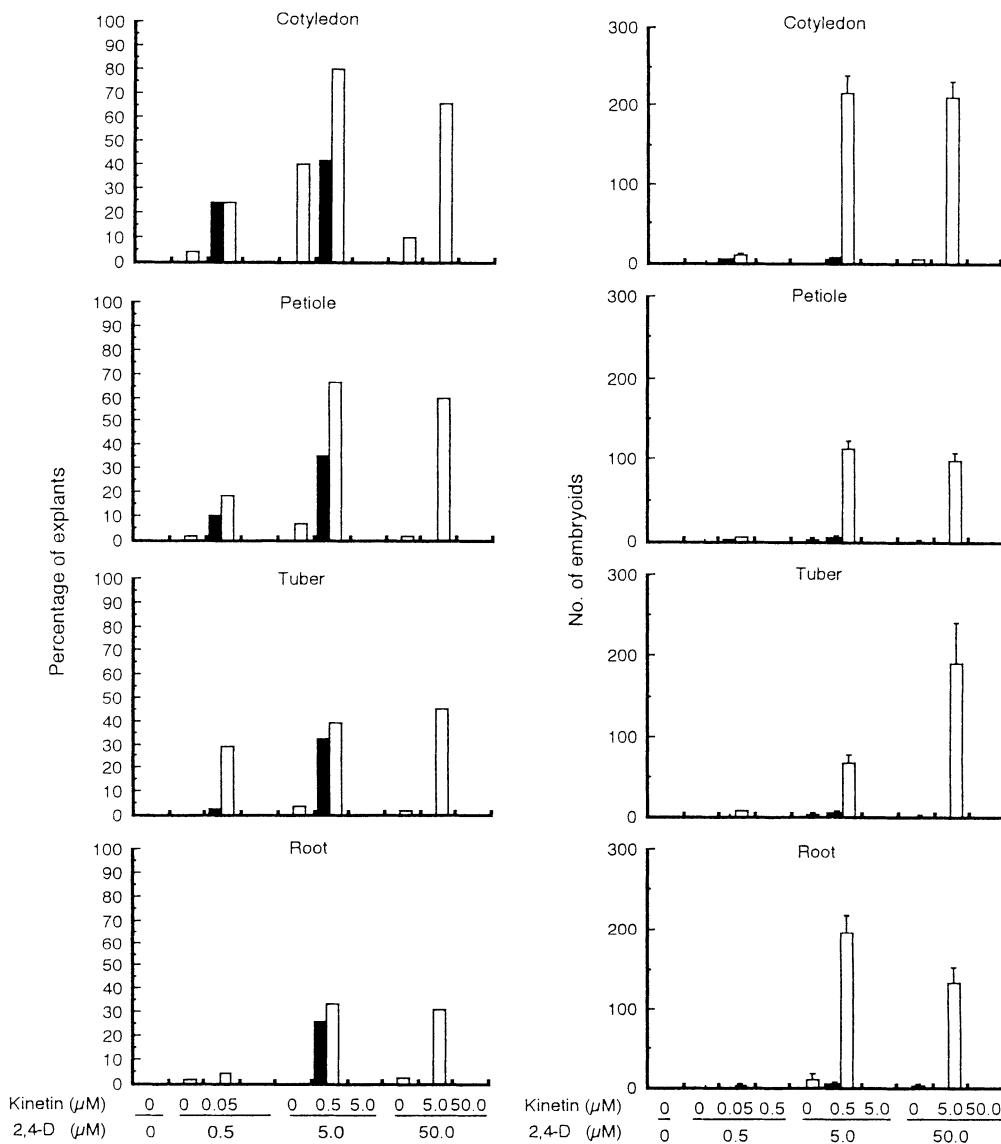


Fig. 1. Effects of plant growth regulators on somatic embryogenesis from aseptic seedling tissues in the cyclamen Anneke. (Takamura et al. 1995). *Left* Percent explants forming embryoids; *right* number of embryoids per organ ■ In primary culture. □ After subculture

The optimal concentration of sucrose in the medium varied according to explant type (Fig. 2). Explants from cotyledons and roots showed the highest embryoid number in the medium with 6% sucrose, while 3% sucrose was optimal for explants from the petioles and tubers.

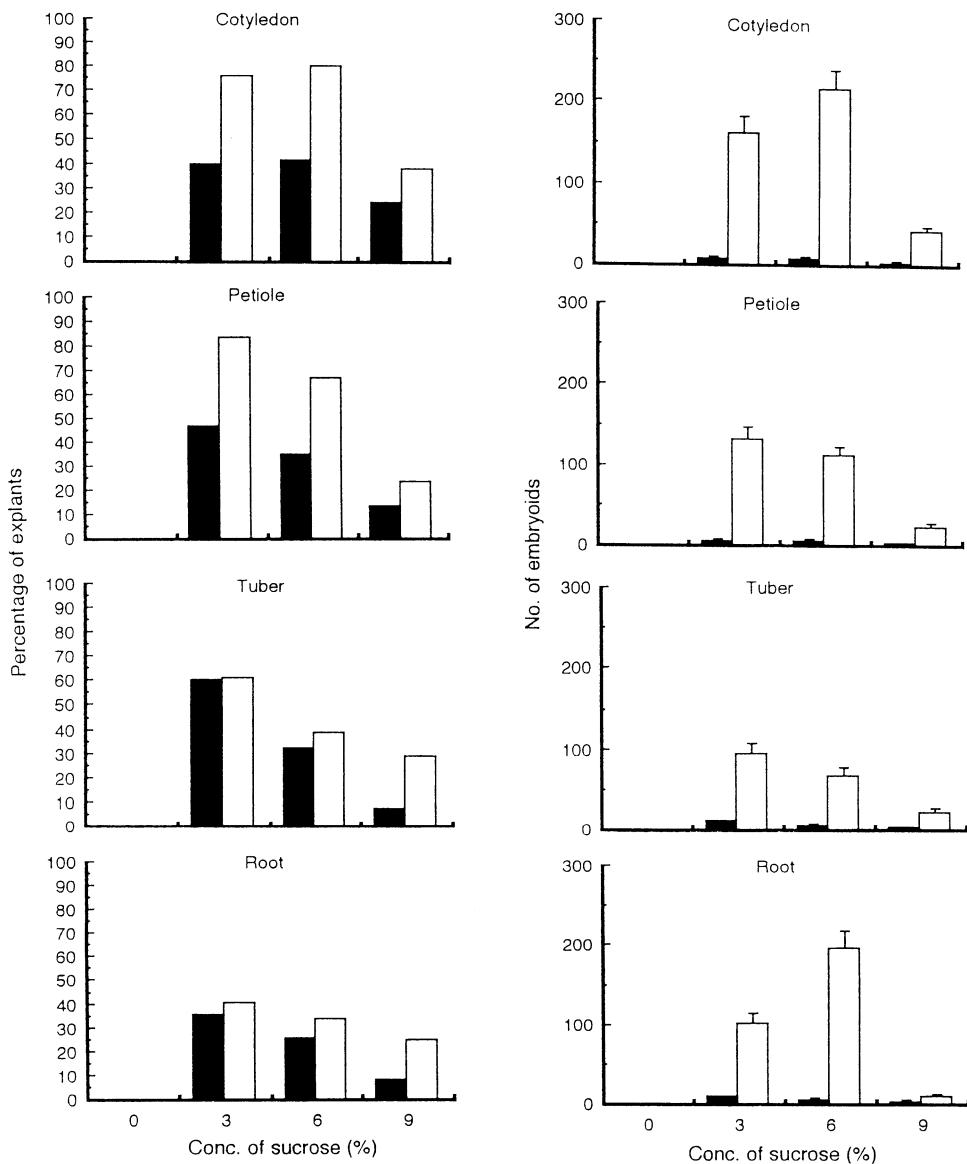


Fig. 2. Effects of sucrose on somatic embryogenesis of the cyclamen Anneke. (Takamura et al. 1995). *Left* Percent explants forming embryos; *right* number of embryos per organ. ■ In primary culture. □ After subculture

3.1.2 Effects of Temperature and Light Condition on Embryogenesis

To investigate the effects of temperature and light on embryogenesis, cultures were maintained at 20, 25, and 30 °C under dark conditions, or cultured in 24-h day length (daylight, about 30 $\mu\text{mol}/\text{m}^2/\text{s}$) at 25 °C. Aseptic seedlings of

Anneke were used. MS media with $5\mu\text{M}$ 2,4-D, $0.5\mu\text{M}$ kinetin, 6% sucrose, and 0.2% gellan gum were used as basic media.

Increasing the temperature from 20 to 30°C enhanced embryogenesis in all types of explants (Fig. 3). Many explants formed embryoids in primary

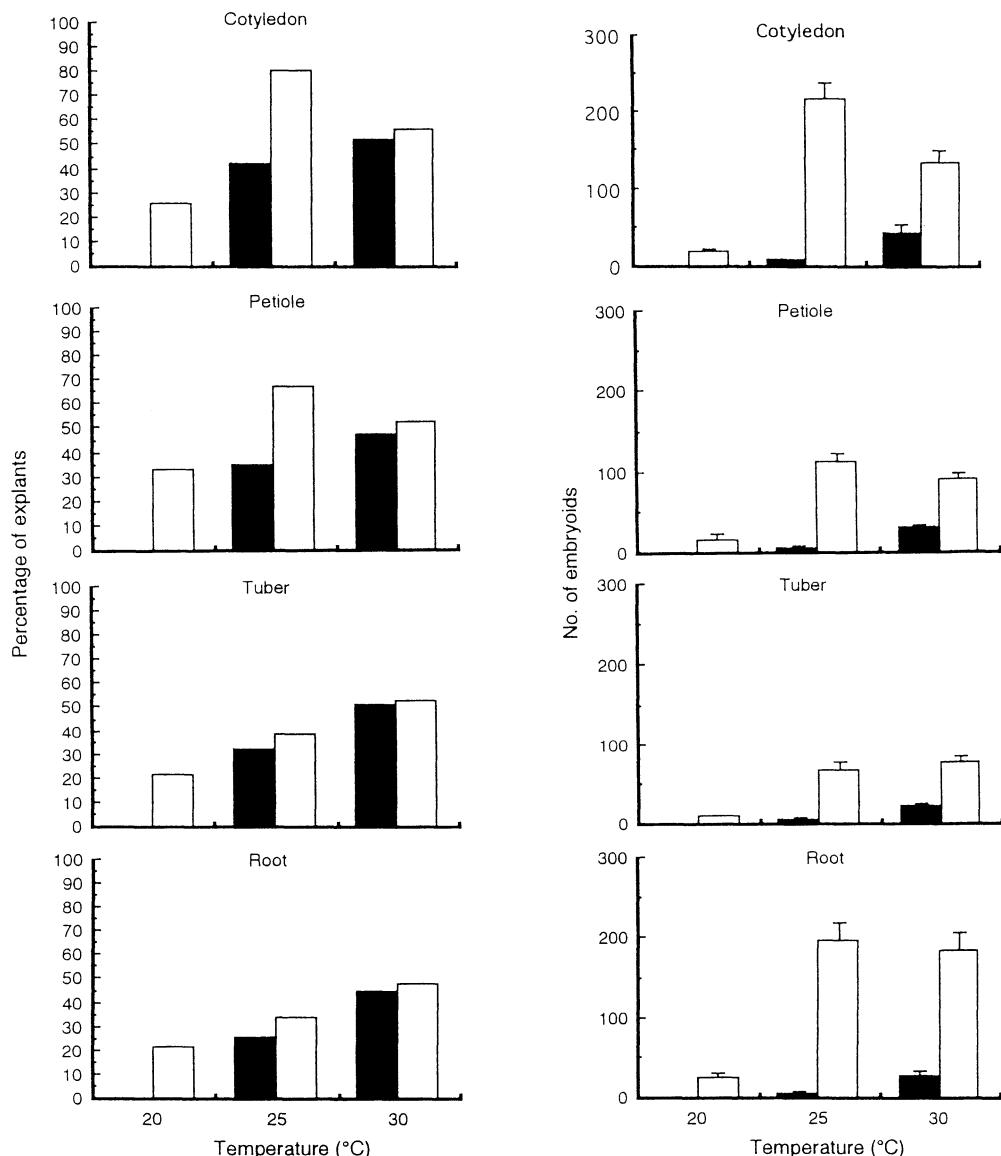


Fig. 3. Effects of temperature on somatic embryogenesis of the cyclamen Anneke. (Takamura et al. 1995). *Left* Percent explants forming embryoids; *right* number of embryoids per organ. ■ In primary culture. □ After subculture

cultures when incubated at 30°C. After subculture, the explants from the cotyledons and petioles, however, showed the highest embryoid number and percentage of explants forming embryoids at 25°C. Other explants showed no significant difference in the number of embryoids produced between 25 and 30°C incubation.

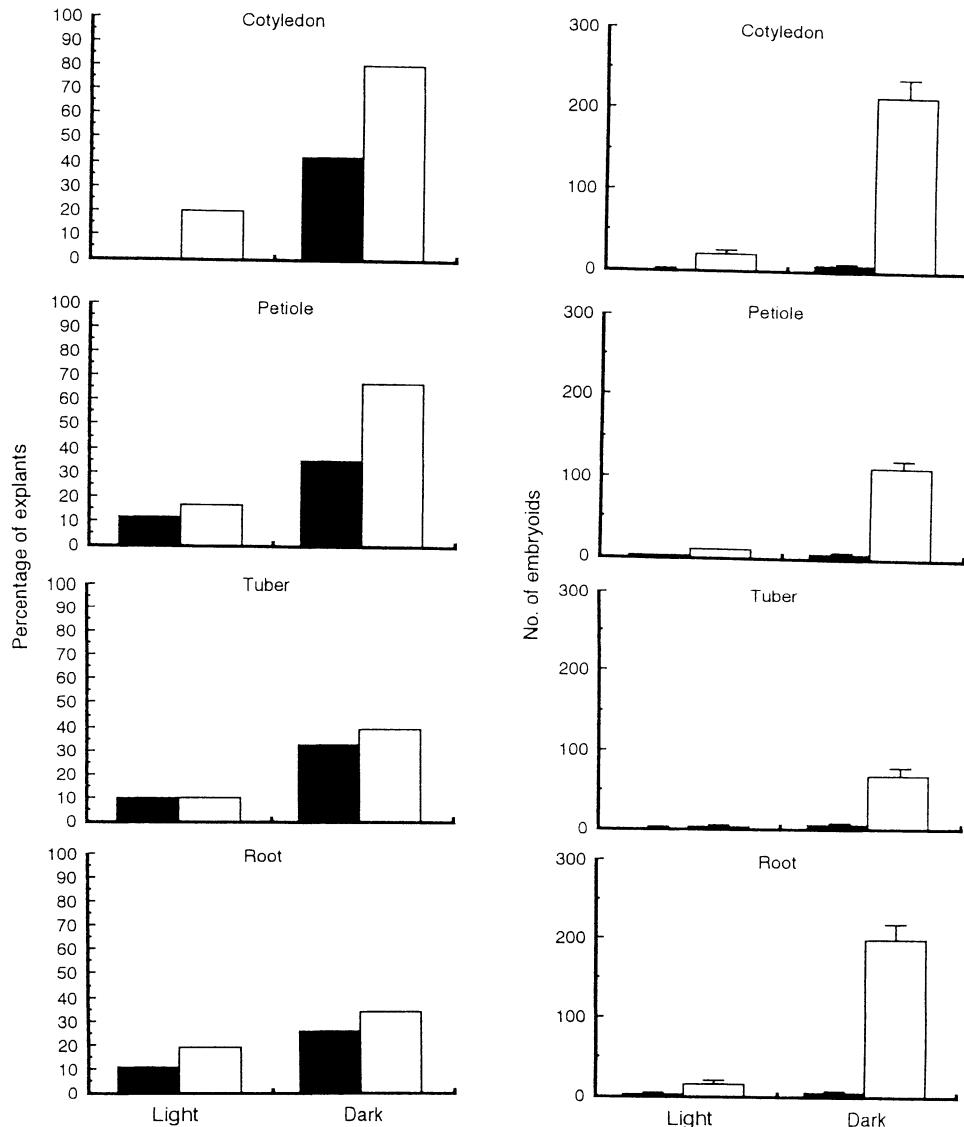


Fig. 4. Effects of light on somatic embryogenesis of the cyclamen Anneke. (Takamura et al. 1995). *Left* Percent explants forming embryoids; *right* number of embryoids per organ. ■ In primary culture. □ After subculture

Kiviharju et al. (1992) reported that germination of somatic embryos was significantly better in the dark than in the light, but they did not mention the effects of light conditions on embryogenic callus formation and somatic embryo differentiation. In this study, a much higher number of embryoids and percentage of embryogenic callus formation was observed in the dark than in the light (24-h day length) in all types of explants (Fig. 4).

From these results, it is suggested that high temperature stimulates embryogenesis, although it is not always effective in some types of explants, and that light inhibits somatic embryoid differentiation as well as germination of embryoids in cyclamen. The effects of other environmental conditions, i.e., humidity, O_2 , CO_2 , etc., on somatic embryogenesis in cyclamen should also be investigated.

3.2 Histological and Morphological Observation of Embryo Differentiation and Growth

For histological observation, embryogenic calli and somatic embryoids were fixed in FAA solution (formalin: acetic acid: 70% ethanol, 1:1:18, v/v), and dehydrated in tertiary butyl alcohol and embedded in paraffin (melting point 58–60°C). Paraffin-embedded material was cut into sections 10 μ m thick, and stained with 0.25% Hidenhein's iron hematoxylin. In all experiments, embryoids were white and formed usually from transparent and friable callus (Fig. 5A). From histological examination, it was observed that embryoids arose from the globular through the heart-shaped stage, as in zygotic embryos. These embryoids had no vascular connection to the embryogenic callus (Fig. 5B). Somatic embryoids transferred to MS medium with 3% sucrose, 0.1 μ M

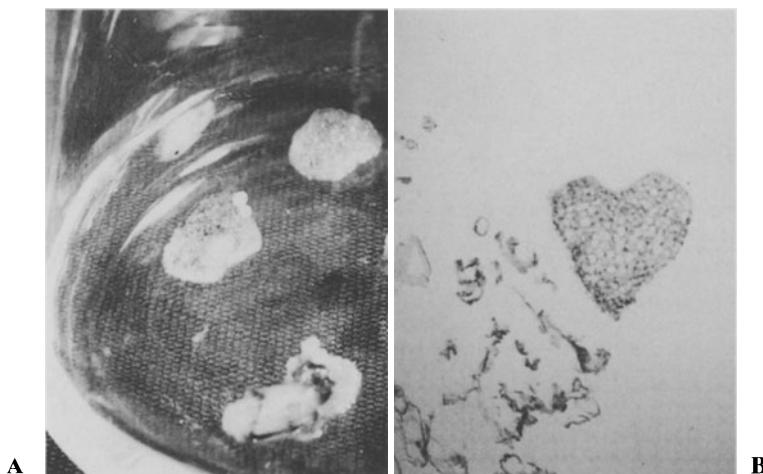


Fig. 5A,B. Embryoids from aseptic seedling tissues. (Takamura et al. 1995). **A** Somatic embryos from transparent and friable callus (in primary culture). *Bar* 2 mm. **B** Heart-shaped somatic embryo (10 μ m section). *Bar* 100 μ m

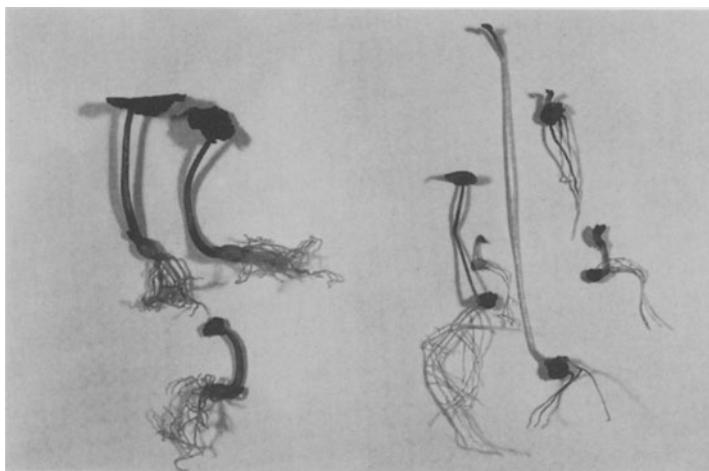


Fig. 6. Plantlets from somatic embryos and seeds. *Bar 1cm.* (Takamura et al. 1995). Left Seedlings from seeds; right young plantlets from embryos



Fig. 7. Flowering plants derived from somatic embryos of *C. persicum* Anneke. *Bar 5cm.* (Takamura et al. 1995)

kinetin, and 0.2% gellan gum showed close similarity to seedlings (Fig. 6). From these observations, it was confirmed that the somatic embryos obtained in this study conformed to the general definition of somatic embryos. Regenerated plantlets were grown in the greenhouse after acclimatization. Approximately 60% of plantlets derived from somatic embryos grew well and flowered in the greenhouse (Fig. 7). No morphological variation was observed, although some of plantlets, which died before anthesis, showed abnormal morphogenesis.

3.3 Varietal Differences in Somatic Embryogenesis

Cotyledons from aseptic seedling of 11 cyclamen cultivars (13 lines) were used as explants. MS media with $5\text{ }\mu\text{M}$ 2,4-D, $0.5\text{ }\mu\text{M}$ kinetin, 6% sucrose, and 0.2% gellan gum were used, and cultures were maintained at 25°C under dark conditions. Subculture and observation of embryogenesis were as mentioned earlier.

Embryogenesis was not observed in the many cultivars examined (Fig. 8). In cultivars where embryogenesis was observed, the percentage of embryogenic callus formation and the number of somatic embryos varied according to the cultivars, as Anneke showed four times more embryogenic callus formation than other cultivars. It seems that there was either a varietal difference in embryogenetic ability or that the optimum conditions for embryogenesis vary according to cultivars in cyclamen.

3.4 Conclusion

All explant types from vegetative organs of aseptic seedling had somatic embryogenetic ability in *Cyclamen persicum* Anneke. Generally, a high percentage of explants forming embryoids and embryo number was recorded in MS medium with $5\text{--}50\text{ }\mu\text{M}$ 2,4-D, kinetin whose concentration was one tenth that of 2,4-D, and 3–6% sucrose. However, the medium composition varied according to the type of explant. It was observed that higher temperature stimulated embryogenesis, but not always, and that light inhibited embryogenesis. Histological and morphological studies showed that the embryoids formed conformed to the general definition of somatic embryos. There was also a varietal difference in somatic embryogenesis.

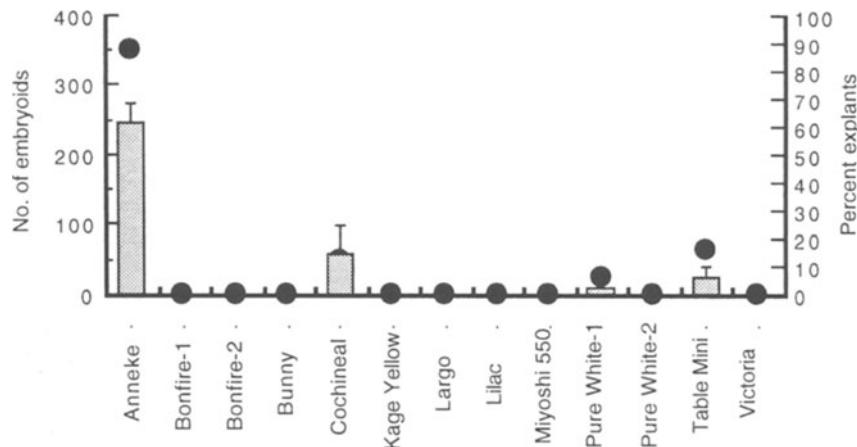


Fig. 8. Varietal difference of callus formation and somatic embryogenesis from cotyledon explants after subculture. (Takamura et al., unpubl.). ● Percent explants forming embryos; ■ No. of embryos per organ

4 Micropagation Via Organogenesis in the Breeding of Yellow-Flowered Cyclamen

Yellow-flowered cyclamen was found in an inbred population of a diploid white-flowered cultivar Pure White, and contains chalcone as main agent of the yellow flower color (Miyajima et al. 1991). It is expected to be useful as material for the breeding of new cultivars, but it needs a long-term process to obtain superior yellow-flowered cyclamen cultivars from the present cultivars. Yellow-flowered plants also seem to be recessive mutants lacking chalcone-flavanone isomerase. Consequently, rapid detection of seedlings predicted to flower yellow and their mass propagation are necessary to produce new yellow-flowered cyclamen and facilitate the breeding of yellow-flowered cyclamen.

4.1 Seedling Selection for Micropagation

In mature plants of yellow-flowered cyclamen mutant, the appearance of the yellow color is not limited to the petals but also appears slightly in leaves and

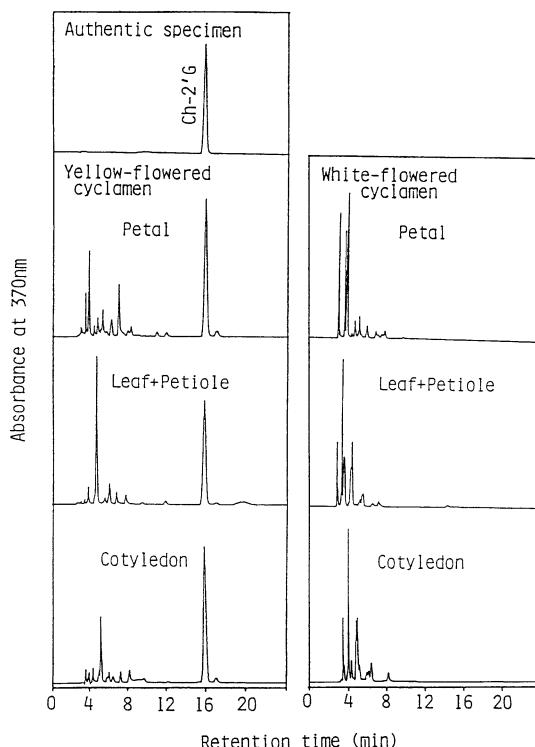


Fig. 9. HPLC profiles of pigment constitutions extracted from various organs in yellow- and white-flowered cyclamen. Ch-2'G Chalcononaringenine 2-glucoside. (Takamura et al. 1993)

leaf petioles. Therefore, in order to determine whether a correlation exists between the pigments in the petals and vegetative tissue, including leaves, leaf petioles of mother plants, and cotyledon of seedlings, their pigment profiles were investigated by high performance liquid chromatography (HPLC). Pigment analysis was made in accordance with the report on the main pigments in yellow-flowered cyclamen petals (Miyajima et al. 1991).

Leaves and leaf petioles of yellow-flowered cyclamen plants appeared yellowish green, whereas those of Pure White were deep green. Particularly the reverse side of the leaves, except on the leaf vein, appeared yellowish green in the former plants, whereas those of the latter showed a red color, due to anthocyanin pigment. In a previous report (Miyajima et al. 1991), the main agent of the yellow color of yellow-flowered cyclamen mutant was identified as chalcononaringenin 2-glucoside. It was not detected in the leaves, leaf petioles, and cotyledon of white-flowered cyclamen Pure White or in the petals. On the other hand, the same vegetative tissue of yellow-flowered cyclamen and the petals contained the same pigment (Fig. 9).

In F_2 offspring obtained from crossing Pure White and yellow-flowered cyclamen, there were yellowish green and deep green seedlings. Young seedlings which had yellowish green leaves were easily distinguished from the others, and yellowish green seedlings had yellow flowers at their anthesis.

From these facts, it seems that seedling selection of yellow-flowered cyclamen is possible at the young seedling stage, and can be useful for the micropagation of yellow-flowered cyclamen cultivars in breeding.

4.2 Micropagation of Selected Seedlings

Wainwright and Harwood (1985) and Hawkes and Wainwright (1987) reported the advantage of micropagation with juvenile tissue in cyclamen because of the potentially high frequency of organogenesis. Furthermore, we previously reported that seedling selection of yellow-flowered cyclamen is possible at the young seedling stage.

We carried out the selection of seedlings expected with yellow petals from F_2 progenies between yellow-flowered cyclamen and other cultivars, and examined their micropagation via organogenesis since it had been difficult to multiply Kage Yellow via embryogenesis (see Sect. 3.3 Varietal Differences, Fig. 8).

4.2.1 Raising of Young Seedlings In Vitro

F_2 seeds crossed between Pure White and yellow-flowered cyclamen were soaked in sodium hypochlorite solution (2% available chlorine) for 10 min and rinsed three times with sterile distilled water. The disinfected seeds were sown on one-third strength MS medium supplemented with 3% sucrose and 0.3% gellan gum. They were incubated at 20°C under dark conditions for 3 weeks followed by 12-h daylength (ca. 30 $\mu\text{mol}/\text{m}^2/\text{s}$). After 7 weeks, young seedlings were obtained.

4.2.2 Multiplication of Selected Seedlings

Young seedlings with a yellowish green cotyledon (especially the reverse side) and yellow roots were selected and divided into cotyledons, leaf petioles, tubers, and roots after 6 weeks' incubation. The cotyledons, leaf petioles, and tubers were divided into five, four, and eight segments, respectively, and the roots were cut into sections 5 to 8 mm in length and cultured.

One-third MS medium containing 3% sucrose and 0.3% gellan gum was used as the basal medium. For examination of the effects of plant growth regulator on shoot formation, various concentrations of 1-naphthalenacetic acid (NAA) and N⁶-benzyladenine (BA) supplemented the basic medium. The cultures were maintained at 20 °C throughout. Cultures were in the dark for the first 35 days, but later in 16-h day length.

The number of adventitious shoots recorded after 60 days of culture is shown in Fig. 10. Adventitious shoot differentiation was observed from the calli, which formed from all explant types except roots. Explants from cotyledons and leaf petioles showed the highest shoot numbers, with 0.1 μ M NAA

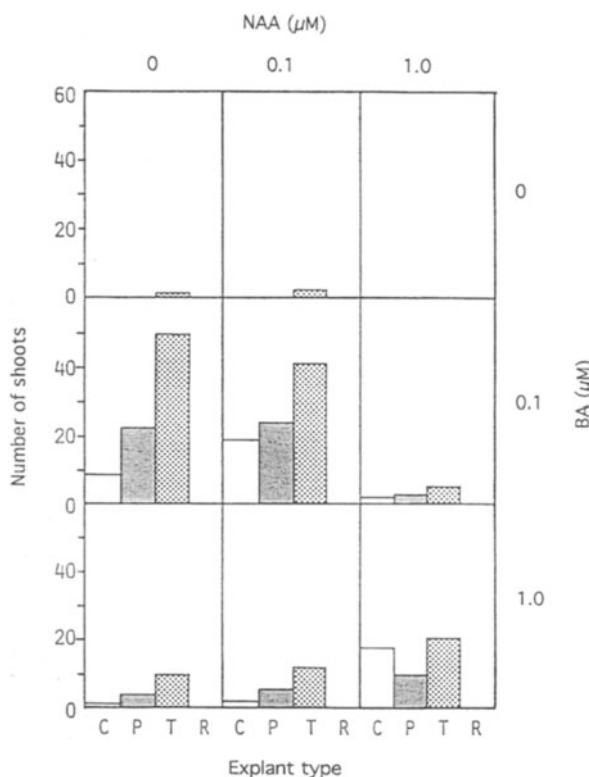


Fig. 10. Number of shoots per mother stock when cultured on 9 different media for 60 days. Explant type: C Cotyledon; P petiole; T tuber; R root. (Takamura et al. 1993)

and $1\text{ }\mu\text{M}$ BA, and those from tubers showed the highest shoot formation with $1\text{ }\mu\text{M}$ BA. The adventitious shoots were transferred to the basic medium containing $0.5\text{ }\mu\text{M}$ NAA for rooting, and most of them differentiated roots.

The calli which were formed during the culture were cut in eight pieces and cultured on the basal medium with $1\text{ }\mu\text{M}$ BA or $0.1\text{ }\mu\text{M}$ NAA and $1.0\text{ }\mu\text{M}$ BA under the same incubation condition as after the removal of the adventitious shoots. Shoot formation was examined after 40 days in the second culture; the results are summarized in Table 1. Adventitious shoots developed again from the calli formed on all explant types examined on the medium which gave highest shoot formation in the first culture, although the percentage of shoot formation decreased slightly in the second culture.

4.2.3 Characteristics of Regenerated Plantlets and Plants

The shoots formed roots when they were transferred to the medium with NAA. The regenerated plantlets from the adventitious shoots showed a yellowish green color on their young leaves and a yellow color on the surface of their roots. From investigation by HPLC, it was proved that they contained the same chalcone pigments as their mother plants.

Approximately 50% of the plantlets planted in soil flowered, and all of them had yellow flower petals. In appearance, there were few differences among the regenerated plants. Wainwright and Harwood (1985) reported that seedling tissue is suitable for in vitro multiplication of cyclamen, in order to eliminate microbial contamination and to obtain genetical uniform plants. Also in our experiments, there was little frequency of contaminations from mother stocks and it seems possible to obtain uniform stocks.

Table 1. Percentage of explants forming shoots and number of shoots per mother stock in primary and second^a culture. (Takamura et al. 1993)

Explant type	Conc. of plant growth regulator (μM)		Percentage of explants forming shoots	No. of shoots per explant	No. of shoots per mother stock
	NAA	BA			
Cotyledon	Pr ^b	0.1	1	70	3.80
	Sc ^c	0.1	1	55.6	1.78
Petioles	Pr	0.1	1	93.8	6
	Sc	0.1	1	87.5	5.71
Tuber	Pr	0	1	90.6	6.25
	Sc	0	1	72.5	6.28
	Sc	0.1	1	93.8	6.84

^aExplants were taken from the callus of primary explants.

^bIn primary culture.

^cIn second culture.

4.3 Conclusion

Mass propagation of yellow-flowered cyclamen, Kage Yellow, via organogenesis was attempted since it had been difficult to multiply Kage Yellow via embryogenesis. It was confirmed that the seedlings of yellow-flowered cyclamen could be multiplied via organogenesis after seedling selection in the breeding cycle. Oohashi et al. (1989) reported that it was possible to obtain about 180 000 plants in six regeneration cycles by using tuber from in vitro disinfected cyclamen seedlings. In this study, we obtained hundreds of plantlets from one seedling in two culture cycles. Thus, this method should be made available for mass propagation of yellow-flowered cyclamen in the breeding cycle.

5 Summary

5.1 Plant Regeneration Via Embryogenesis from Aseptic Seedling Tissue

In *Cyclamen persicum* Anneke, explants from the various vegetative organs of aseptic seedlings formed embryoids. The optimal responses were recorded in MS medium enriched with $5.0\mu\text{M}$ 2,4-D, $0.5\mu\text{M}$ kinetin, and 3–6% sucrose. Embryogenesis was enhanced at a higher temperature of 25–30°C. On the other hand, light inhibited embryogenesis. Histological and morphological studies confirmed that the embryoids were, in fact, somatic embryos. There was also varietal difference in somatic embryogenesis in cyclamen.

5.2 Micropropagation via Organogenesis in the Breeding of Yellow-Flowered Cyclamen

Seedlings expected to be yellow-flowered could be selected by their color in F_2 progenies crossed between Pure White and Kage Yellow, and their micropropagation was examined. The most suitable explants were the tuber tissues, which gave the highest shoot formation with the $1\mu\text{M}$ BA. Approximately 50% of the plantlets planted into soil flowered, and all of them had yellow flower petals. In this study, we obtained hundreds of plantlets from one seedling in two culture cycles. Thus, this method should be made available for mass propagation of yellow-flowered cyclamen in the breeding cycle.

6 Protocol

From the facts above, we may conclude that the best methods for plant regeneration via embryogenesis and for micropropagation via organogenesis for the breeding of yellow-flowered cultivars are as follows.

6.1 Plant Regeneration Via Embryogenesis from Aseptic Seedling Tissue

The explants for culture should be obtained from the 7-week-old aseptic seedlings cultured on one-third MS medium containing 3% sucrose at 20°C in the dark for 3 weeks followed by 16-h day length.

Each explant should then be cultured on MS medium containing 3% (petiole and tuber explants) or 6% sucrose (cotyledon and root explants) and supplemented with 5 μ M 2,4-D and 0.5 μ M kinetin (cotyledon, petiole, and root explants) or with 50 μ M 2,4-D and 5 μ M kinetin (tuber explants) at 25°C (or 30°C in tuber explants) in the dark. For embryogenesis, calli from each explant should be transferred to MS medium without plant growth regulators. To obtain plantlets, it is desirable for somatic embryos to be replanted onto MS medium with 3% sucrose and 0.1 μ M kinetin.

6.2 Micropagation Via Organogenesis for Breeding Yellow-Flowered *Cyclamen*

Young aseptic seedlings with a yellowish green cotyledon (especially the reverse side) have to be selected from F_2 progenies crossed between yellow-flowered cultivars and others. Each organ except roots can be used as explant, but tuber tissues are most suitable.

For shoot formation, the tuber explants, divided into eight segments, should be cultured on one-third MS (containing 3% sucrose) medium with 1 μ M BA at 20°C in the dark for the first 35 days followed by 16-h daylength. In order to obtain many shoots, the calli formed during the culture should be cut into eight pieces, which should be cultured on one-third MS (containing 3% sucrose) with 1 μ M BA or 0.1 μ M NAA and 1 μ M BA under the same incubation conditions as after the removal of the adventitious shoots. The adventitious shoots should be transferred to one-third MS (containing 3% sucrose) with 0.5 μ M NAA, to obtain the plantlets.

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I.9 Micropagation of *Daphne* L.

T.R. MARKS¹

1 Introduction

1.1 Botany, Distribution, and Importance

The genus *Daphne* L. (Thymelaeaceae family) contains approximately 70 species distributed across Europe and temperate and subtropical Asia, with a few representatives in North Africa. All species can be classified as shrubs, and vary in stature between the prostrate *D. jasminea* (eastern Mediterranean) and the upright *D. bholua* (eastern Himalayas), which can grow to over 3m. *Daphne* plants are grown for their ornamental value in Europe, New Zealand (Christie and Brascamp 1989) and the USA (Gaschk 1989), where they are noted particularly for the fragrance of their flowers, often borne in early spring when few other shrubs are in bloom, and for their generally dwarf growth habit. However, their commercial availability is limited due to their slow growth, and the protracted development of a saleable plant of good form in the nursery. Where they can be readily propagated by seed (*D. mezereum*, *D. laureola* and *D. pontica*) or by cuttings (*D. x burkwoodii* and *D. odora* clones and *D. cneorum* forms) plants are more readily available (Brickell and Mathew 1976). In general, most species are currently underexploited.

Flowers are borne either singly, in short racemes, or in apical or axillary clusters, with many appearing in spring in the UK (Fig. 1), and some having repeat flowering in the autumn. The often fragrant flowers lack petals and consist of a fleshy calyx tube with four spreading lobes which can be greenish white, pink, purple, yellow or orange in colour. Leaves are either sessile or shortly stalked and generally alternate. Exceptions are *D. genkwa*, in which most leaves are opposite, and *D. aurantiaca*, in which all are. They can be evergreen, deciduous or partly evergreen. Even different clones of the same species can be both evergreen and deciduous in the UK, caused by the collection and introduction of different ecotypes from within their distribution range.

Daphne are also used for the production of hand-made paper in the Himalayas. In Nepal, the bark fibres of *D. bholua* and *D. papyracea* are used, and collectively these species and the paper they produce are known as Lokta (Jeanrenaud 1984). In Towang in the northeast Himalayas, *D. cannabina* is

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Fig. 1. Flowers of *Daphne odora* cv. *Aureo-marginata*

also used, and produces a high-endurance and grease-proof paper (Saikia et al. 1972). In both instances, there is concern that the natural resource of these species is being overexploited, and more consideration should be given to replanting programmes (Jeanrenaud 1985).

The chemical constituents of various parts of *Daphne* plants have attracted much attention due particularly to both their medicinal and poisonous properties. Phenolics from the flowers and buds of *D. genkwa* have purgative, diuretic and expectorant effects (Nikaido et al. 1987), while a diterpene (genkwadaphnin) from the same plant is reported to have anti-leukaemic properties (Kasai et al. 1981). *D. mezereum* has caused poisoning in cattle (Pernthaner and Langer 1993), and roots of *D. gnidium* have been used by poachers in southern Italy to poison fish (Carosielli et al. 1991), while *D. odora* root and bark constituents have been shown to have antifungal, insecticidal (Inamori et al. 1987) and nematicidal (Kosigo et al. 1976) properties. It is also reported that essential oils have been distilled from the dried leaves of *D. oleoides* (Topcuoglu 1948).

1.2 Conventional Propagation and Need for Micropropagation

Daphne can be raised by a variety of means depending upon the species, the intended use of the resultant plants, and the availability of material for propagation.

Propagation by seed is possible, but many cultivated species do not set seed freely. Where available, it is best collected before the berry matures and planted immediately to ensure a high germination rate the following spring; if the seed is left to mature, germination may be staggered over 2–3 years (Lamb

and Nutty 1986). Some, but not all, seeds require stratification and should not be stored dry because viability is appreciably reduced.

Layering is often used with established *Daphne* plants, especially where facilitated by a prostrate habit (Lamb and Nutty 1986). Shoots should be pegged to the ground in early spring, and rooting can be assisted by wounding the underside of the shoot and treating with auxin.

Grafting is used where seed is generally unavailable or cuttings root poorly. It is also used to hasten shoot development and early flowering by grafting a slow-growing scion onto a vigorous stock. *D. mezereum* (deciduous) is often used as a rootstock for both deciduous and evergreen scions, and *D. acutiloba* or *D. longilobata* (both evergreen) only for evergreen scions (Brickell and Mathew 1976; Lamb and Nutty 1986).

Leafy cuttings taken between June and August have been successful for a wide range of species and cultivars. Brickell and Mathew (1976) found that more mature cuttings generally require auxin application (0.1–0.8% w/w indolebutyric acid, IBA, in talc), but young cuttings less so (Brickell and Mathew 1976), whereas other reports showed that there was no clear advantage in using an auxin treatment (Lamb and Nutty 1986; Bruckel 1989). The rooting compost must be free-draining and several have been used: peat:sand/grit (2:1; Lamb and Nutty 1986), peat:pumice (1:1; Ripphausen 1990) and perlite:vermiculite (2:1; Bruckel 1989). Propagation in an open mist environment has proved successful for a range of species (Lamb and Nutty 1986; Ripphausen 1990), but the overwetting that can occur caused basal stem rot (Bruckel 1989). Rooting in *D. odora* var. *rubra* was improved by using an enclosed mist system, where variation in relative humidity was reduced (Richards 1985). The type of cutting on a stockplant shoot can also affect rooting and subsequent growth. Ripphausen (1990) found that nodal cuttings produced more roots and earlier lateral shoot growth than did terminal cuttings.

Currently, the major need for clonal propagation is to produce ornamental plants for markets primarily in Europe, Australasia and in the USA. Although conventional cutting techniques have been well developed for a small range of species and clones, the growth of many others is too slow to provide a commercially viable source of material (Brickell and Mathew 1976). *Daphne* plants also act as hosts to a range of horticulturally important viruses. Sweet et al. (1979) reported that cv. Somerset and cv. Somerset Gold Edge, and *D. odora* were variously infected with cucumber mosaic, arabis mosaic and raspberry ringspot virus, and Cohen (1977) showed that *D. x burkwoodii* was also infected with *Daphne* virus S and *D. cneorum* with *Daphne* virus X. The successful use of thermotherapy and meristem tip dissection to eliminate viruses from the *D. x burkwoodii* clones demonstrates their potential as a technology for establishing high health status nuclear stock.

The use of tissue culture to produce *Daphne* plants for their medicinal properties has not been discussed in the literature, but is a prospect for the future. The need to replant *D. bholua* and *D. papyracea* for paper-making in Nepal has been identified, and micropagation could assist in their production.

2 Brief Review of In Vitro Studies

The literature published to date on *Daphne* tissue culture concentrates mainly on *D. x burkwoodii*, *D. odora*, their varieties and cultivars. Early reports showed its use in attempts to eliminate viruses in both New Zealand (Cohen and Le Gal 1976) and in the UK (Sweet et al. 1979). Reinfection with a range of viruses can occur quickly after thermotherapy, and tissue culture has been considered a useful alternative technology to protecting in vivo stock. Interest in their ornamental value led to the culture of *D. cneorum*, *D. laureola*, *D. mezereum*, *D. pontica*, *D. retusa* and *D. tangutica* (Constantine et al. 1982), and a range of species and clones have been commercially available in the UK (Table 1).

2.1 Culture Initiation

Aseptic dissection has been used to obtain axenic cultures following thermotherapy (Cohen and Le Gal 1976; Sweet et al. 1979), or as an alternative to surface sterilization (Constantine et al. 1980), although surface sterilization of apical or axillary buds was successfully achieved using sodium hypochlorite and washing in sterile deionized water (e.g. Marks and Myers 1992).

Several authors have reported the need to initiate cultures on media containing high concentrations of plant growth regulators (PGRs), then to reduce these for sustained axillary shoot multiplication. Cohen and Le Gal (1976) successfully initiated cultures of *D. x burkwoodii* using 1 mg/l kinetin (kin), later reduced to 0.3 mg/l kin, plus 0.1 mg/l naphthaleneacetic acid (NAA), to avoid shoot and leaf distortion. Similarly, *D. odora* cultures were initiated on 1 mg/l NAA and 0.3 mg/l kin, with NAA later reduced to 0.3 mg/l. Schum and Muller (1987) initiated cultures of a *D. cneorum* hybrid on 0.5–1 mg/l 6-benzyladenine (BA), 0.2 mg/l IBA and 0.1 mg/l gibberellic acid (GA₃), and this was reduced to 0.1 mg/l BA and 0.05 mg/l IBA with the same concentration of GA₃, after the first subculture. Marks and Myers (1992) initiated both *D. cneorum* and cv. Aureo-marginata on 5 µM BA, reduced to 0.5 and 1 µM respectively after the first subculture.

Marks and Myers (1992) reported distinct topophytic effects on in vitro shoot growth and rooting. Only the apex and the most distal axillary buds of cv. Aureo-marginata stockplant shoots developed in vitro. The same trend was apparent in *D. cneorum*, but lower axillary buds survived and grew more slowly than apical ones in vitro. Shoots derived from the more proximal explants rooted less well in vitro. This diversification in developmental potential was long-lived, and was expressed in the ex vitro branching habit of cv. Aureo-marginata plants.

Table 1. *Daphne* species, varieties and cultivars which have been commercially microppropagated in the UK

<i>D. arbuscula</i>	<i>D. cneorum</i> variegata	<i>D. odora</i>
<i>D. bholua</i>	<i>D. collina</i>	<i>D. odora</i> Aureo-marginata
<i>D. bholua</i> Gurkha	<i>D. genkwa</i>	<i>D. odora</i> var. <i>leucantha</i>
<i>D. bholua</i> Jacqueline Postill	<i>D. x houtteana</i>	<i>D. odora</i> var. <i>rubra</i>
<i>D. blagayana</i>	<i>D. laureola</i> ssp. <i>philippi</i>	<i>D. odora</i> Walburton
<i>D. x burkwoodii</i>	<i>D. longilobata</i> Peter Moore	<i>D. tangutica</i>
<i>D. x burkwoodii</i> Somerset	<i>D. mezereum</i>	
<i>D. cneorum</i> Eximia	<i>D. x napolitana</i>	

See Brickell and Mathew (1976) for nomenclature and *Acknowledgments* for information on commercial production.

2.2 Axillary Shoot Culture

Murashige and Skoog (1962) MS medium has been used to grow axillary shoot cultures of *D. x burkwoodii* (Cohen and Le Gal 1976; Sweet et al. 1979), *D. odora* (Cohen and Le Gal 1976) and *D. blagayana* (Leifert et al. 1992), Linsmaier and Skoog (1965) LS medium for *D. cneorum* (Marks and Myers 1992), while Woody Plant Medium (WPM, Lloyd and McCown 1981) was used to culture a *D. cneorum* hybrid (Schum and Muller 1987). Kinetin and BA have both been used to induce shoot production in these species and hybrids, often in addition to NAA or IBA respectively, but auxin can be omitted (Marks and Myers 1992). Shoot elongation in *D. odora* was enhanced by 1 mg/l GA₃ (Cohen 1977), and in a *D. cneorum* hybrid by 0.1 mg/l GA₃ (Schum and Muller 1987; Table 2).

Shoot multiplication rates varied with species. Cohen and Le Gal (1976) reported a five-fold increase per subculture for *D. x burkwoodii*, whereas Marks and Myers (1992) reported a three-fold increase for cv. Aureo-marginata. Increased BA improved shoot numbers of cv. Aureo-marginata (Marks and Myers 1994), but the fact that several authors report the need to reduce PGRs after initiation to avoid aberrant growth suggests that *Daphne* species are very sensitive to the concentrations used. *D. blagayana* multiplication rates were also affected by medium pH, being lower with an initial pH of 3.5 than with one of pH 5.6 (Leifert et al. 1992).

Photoperiods of 16h are commonly reported, for example provided by either white fluorescent lamps with an irradiance of 11 W/m² (400–700 nm photosynthetically active radiation, PAR) for *D. x burkwoodii* and *D. odora* (Cohen and Le Gal 1976), or alternatively GroLux fluorescent lamps with a photon flux density of 48 μmol/m²/s (400–700 nm, PAR) for *D. cneorum* and cv.

Table 2. Reported use of plant growth regulators for axillary shoot cultures of *Daphne* clones

Clone	Plant growth regulator			Reference
	Auxin	Cytokinin	Gibberellic acid	
<i>D. blagayana</i>	–	0.44 µM BA		Leifert et al. (1992)
<i>D. x burkwoodii</i>	0.1 mg/l NAA	0.3 mg/l Kinetin		Cohen and Le Gal (1976)
cv. Somerset	–	2.5 µM		Sweet et al. (1979)
cv. Somerset Gold Edge		Kinetin		
<i>D. cneorum</i>	–	0.5 µM BA		Marks and Myers (1992)
<i>D. cneorum</i> hybrid	0.05 mg/l IBA	0.1 mg/l BA	0.1 mg/l GA ₃	Schum and Muller (1987)
<i>D. odora</i>	0.3 mg/l NAA	0.3 mg/l Kinetin		Cohen and Le Gal (1976)
var. <i>leucantha</i>	1 mg/l	0.3 mg/l		Cohen (1977)
var. <i>rubra</i>	NAA	Kinetin		
cv. Aureo-marginata	–	1 µM BA		Marks and Myers (1992)

Aureo-marginata (Marks and Myers 1994). Temperatures were maintained at $25 \pm 2^\circ\text{C}$ in both cases.

2.3 Rooting

Two basic methods have been used to root *Daphne* shoots. Either both induction and expression were performed in vitro, or shoots were direct-rooted into compost in a high humidity environment.

Cohen and Le Gal (1976) showed that low medium concentrations of NAA failed to root *D. x burkwoodii* while high concentrations induced callus and distorted roots, whereas dipping shoots in 200 mg/l NAA for 10 min resulted in up to 70% rooting on a hormone-free (HF) medium. Using a longer dipping time of 1–2 h, a similar proportion of *D. odora* var. *leucantha* were rooted (Cohen 1977). Li and Chen (1986) obtained 80–90% rooting for *D. odora* on MS medium supplemented with 1 mg/l IBA, 1 mg/l NAA and 1–2 mg/l phloridzin, without transfer to an HF medium. Christie and Brascamp (1989) used 0.5 mg/l NAA and 0.5 mg/l IBA as a dip, also supplemented with 2 mg/l phloridzin, to root *D. odora* var. *leucantha* and var. *rubra* (65%) in peat:pumice (50:50). However, raising the auxins alone to 5 mg/l gave a similar level of rooting. Schum and Muller (1987) could root a *D. cneorum* hybrid in WPM supplemented with 10 mg/l IBA (70–80%), and Leifert et al. (1992) rooted *D. blagayana* on half-strength WPM supplemented with 2.46 µM IBA in vitro, while *D. cneorum* could be rooted ($\leq 96\%$) following induction on half-strength LS supplemented with 30 µM IBA for 7 days and expression on HF quarter-strength LS (Marks and Myers 1994). Sweet et al. (1979), were

unable to root *D. odora*, cv. Somerset and cv. Somerset Gold Edge on MS medium supplemented with 5 μ M NAA or IBA, but cv. Somerset could be direct-rooted in perlite following a basal dip in 0.2% w/w IBA in talc. cv. *Aureo-marginata* could be rooted (65%) in a peat-based compost using the same method (Marks and Myers 1994).

2.4 Acclimatization

Cohen and Le Gal (1976) first acclimatized rooted shoots of *D. x burkwoodii* in Jiffy 7 peat pots under glass jars in the culture room for 2 weeks followed by a further week in the glasshouse under intermittent mist. Alternatively shoots of *D. odora* var. *leucantha* and var. *rubra* were acclimatized in pumice:peat (70:30) on capillary matting under a high humidity tent for 8 weeks (Christie and Brascamp 1989). Marks and Wiltshire (1985) showed that earlier regrowth of *D. retusa* was promoted by weaning under wet fog (Agritec), where leaf wetting and relative humidity (RH) were less variable than other environments. Alternatively, fully acclimatized plants of cv. *Aureo-marginata* were achieved by rooting in a peat-based compost in a dry fog environment (Lucas Dawes Sonicore, operating at 0.4 °C wet bulb depression) for 4 weeks, followed by 7 days at >90% RH and a further 7 days at 60–80% RH in polythene-covered tents (Marks and Myers 1994).

3 Micropropagation Experiments

3.1 Controlling Culture Initiation

Growing stockplants under protection, such as in a glasshouse, helps reduce the micro-floral population and raises the efficiency of surface sterilization procedures (Debergh and Maene 1981). Two sterilization methods were used with explants from cv. *Aureo-marginata* plants grown under glass. The first used a sodium hypochlorite solution containing 0.7% w/v available chlorine (Marks and Myers 1992), and the second used both mercuric chloride (0.5% w/v) and calcium hypochlorite (1% w/v available chlorine) which had been developed for use with field-raised stock plants (Marks and Simpson 1990; Table 3). Omission of mercuric chloride from the second technique reduced the number of necrotic cultures, and the same concentration of sodium hypochlorite was used successfully to initiate axenic cultures of *D. cneorum* and *D. collina*.

The need initially to grow *Daphne* explants at elevated concentrations of PGRs (Sect. 2.1), especially cytokinin, to stimulate axillary shoot growth induced hyperhydricity in *D. cneorum* cultures supplemented with 5 μ M BA (Fig. 2). Hyperhydricity was subsequently eliminated by transfer to 0.1 μ M BA, but normal shoot cultures were not obtained until at least the fifth subculture (Fig. 3). This can be averted by initiating multinodal explants of *D. cneorum* on LS medium supplemented with 1 μ M BA and 0.005 μ M

Table 3. Sterilization of *Daphne odora* Aureo-marginata explants using alternative techniques (T.R. Marks, unpubl.)

	0.7% av. chlorine (sodium hypochlorite)	0.5% mercuric chloride and 1% av. chlorine (calcium hypochlorite)	LSD (P = 0.05)
No. of explants used	77	62	—
Viable cultures (%)	90	56	14.1
Necrotic cultures (%)	3	32	12.2
Contaminated cultures (%)	8	11	10.0

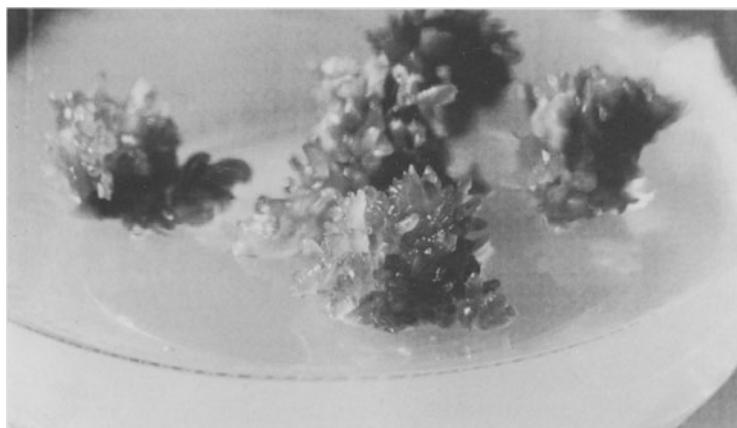


Fig. 2. Development of hyperhydricity in 4-month-old axillary shoot cultures of *Daphne cneorum* growing on LS medium supplemented with 5 μ M BA



Fig. 3. Normal axillary shoot cluster of *Daphne cneorum* grown on medium supplemented with 0.1 μ M BA. Culture is 4.5 years old

thidiazuron (TDZ). Thidiazuron has a strong cytokinin-like action on axillary buds (Mok et al. 1982), and can also act in concert with BA (Marks and Simpson 1994). Transfer to 0.1 μ M BA at the first subculture resulted in non-hyperhydric normal shoots appearing at the end of that subculture. *D. collina* also required to be initiated as multi-nodal explants on LS medium supplemented with 1 μ M BA, but the addition of 0.005 μ M TDZ caused necrosis. As with *D. cneorum*, single-node explants of *D. collina* could not be cultured.

3.2 Factors Affecting Shoot Proliferation

For axillary shoot culture LS medium was used throughout, supplemented with only BA (0.1–1 μ M), and adjusted to pH 5.2 before the addition of a gelling agent and autoclaving. Only *D. tangutica* had been cultured for sustained periods on a different medium following an unpublished report suggesting the use of WPM for shoot multiplication prior to rooting on LS medium. However, under our conditions, better growth was achieved with LS medium. Table 4 summarises the BA concentrations used, and the type and level of light used (16-h photoperiod) for a range of species and cultivars. The concentration of BA used shows some taxonomic relationships. *D. x burkwoodii* is a hybrid of *D. cneorum* (0.1 μ M), whilst *D. odora*, *D. retusa* and *D. tangutica* (1/0.5 μ M) are closely related, as are *D. laureola* and *D. pontica* (1 μ M). *D. collina* (0.5 μ M) belongs to a separate subsection within the genus (Brickell and Mathew 1976). Subcultures of 4 weeks were used for *D. x burkwoodii*, *D. collina* and *D. cneorum*, and 6 weeks for the rest. To restrict variability, similar shoot types must be used (Marks and Myers 1994), and either an intact or a decapitated shoot (1.5–2 cm long) can be transferred to fresh medium.

Only medium supplemented with cytokinin has been necessary to stimulate axillary shoot growth, but concentration-related increases in shoot num-

Table 4. Conditions used for the axillary shoot culture of various *Daphne* species and cultivars in vitro

Clones	Medium	BA concentration (μ M)	Light source	Temp. $\pm 2^\circ\text{C}$
<i>D. x burkwoodii</i> ^b	LS	0.1	OWW	22
cv. Somerset ^b	LS	0.1	OWW	22
<i>D. cneorum</i> ^a	LS	0.1	TGL	25
<i>D. collina</i> ^c	LS	0.5	TGL	25
<i>D. laureola</i> ^b	LS	1	OWW	22
cv. Aureo-marginata ^a	LS	1	TGL	25
<i>D. pontica</i> ^b	LS	1	OWW	22
<i>D. retusa</i> ^b	LS	1	OWW	22
<i>D. tangutica</i> ^c	LS, WPM	0.5	PC84	25

Light sources (400–700 nm) were OWW, Osram warm white (88 $\mu\text{mol/m}^2/\text{s}$); TGL, Thorn GroLux (48 $\mu\text{mol/m}^2/\text{s}$) and PC84, Phillips Colour 84 (100 $\mu\text{mol/m}^2/\text{s}$).

^a Marks and Myers (1992).

^b Marks (unpubl.).

^c Kubba (unpubl.).

bers are limited by the onset of hyperhydricity observed in many subjects if kept on media at concentrations above $1\text{ }\mu\text{M}$ BA. However, over a single subculture, a dose response can be demonstrated in *D. cneorum* shoot culture. Shoot production increased with increasing BA concentration, rising to a maximum at $1\text{ }\mu\text{M}$. Fresh weight also reached a maximum at $1\text{ }\mu\text{M}$, but callus rather than shoots was produced at higher concentrations (Fig. 4). A common practice to increase shoot numbers is to remove the apex at

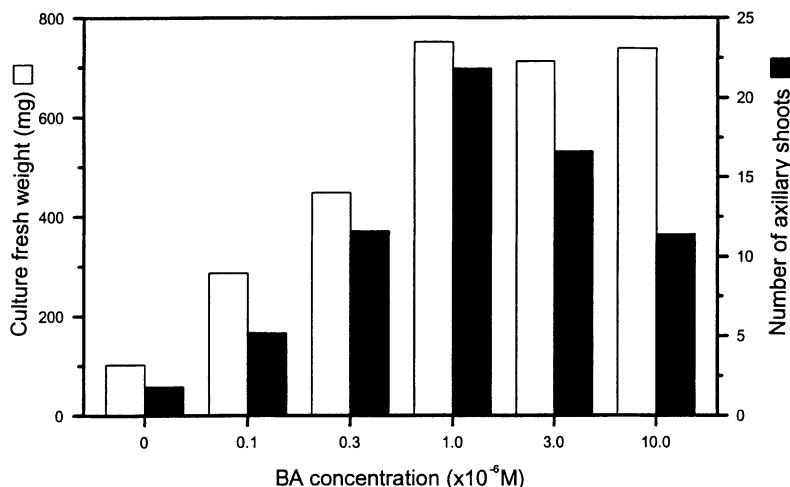


Fig. 4. Effect of BA concentration on axillary shoot proliferation in cultures of *Daphne cneorum*. Cultures are 3.5 years old

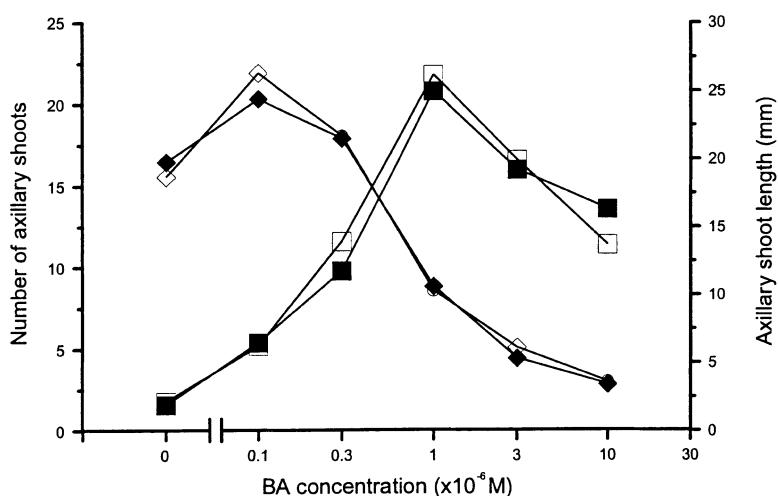


Fig. 5. Effect of BA concentration on shoot proliferation (with □, without ■ tips) and elongation (with ◇, without ◆ tips) in *Daphne cneorum*. Cultures are 3.5 years old

Table 5. Effect of different gelling agents on the growth of *Daphne* axillary shoots over one subculture (J. Kubba, unpubl.)

	Technical (0.7%)	Purified (0.8%)	Agargel (0.5%)	Gelrite (0.25%)	LSD (<i>P</i> = 0.05)
<i>cv. Aureomarginata</i>					
No. of laterals	2.5	1.3	3.0	0.2	0.37
Length of explanted shoot (mm)	36.1	31.8	32.2	6.4	2.26
Mean axillary shoot length (mm)	21.8	19.5	18.4	20.3	2.21
<i>D. tangutica</i>					
No. of laterals	0	0	0	—	—
Length of explanted shoot (mm)	22.6	44.5	27.8	—	9.31

subculture. In *D. cneorum* cultures this made no difference to shoot numbers, despite the low BA concentration. Maximum shoot length was attained at 0.1 μ M BA, and these proved the most suitable for further subculture and rooting (Fig. 5).

Often overlooked as a factor affecting axillary shoot growth is the gelling agent used to solidify the medium. Using Technical and Purified agar (Oxoid), Agargel (Sigma) and Gelrite (Scott), at concentrations giving the same gel strength, different gelling agents were shown to affect multiplication and shoot elongation in cultures of *cv. Aureo-marginata* and *D. tangutica* (Table 5). Gelrite, in particular, induced hyperhydricity and necrosis in both.

3.3 Root Induction and Acclimatization

A principle feature reported in the literature on root induction in vitro is the use of comparatively high concentrations of auxin, applied either through continual contact with the microcutting throughout rhizogenesis, or as a pulse before planting in a medium lacking growth regulators. Table 6 illustrates the use of this latter system where 1.5 cm shoots were treated with auxin (IBA) for 7 days before transfer to a HF-free medium to allow root expression (Fig. 6). It is often beneficial to lower mineral salt concentration for root expression, but the same salt medium as used for shoot proliferation can be used for root induction. *D. cneorum* is an exception to this, and lower medium salt concentrations are used throughout rooting. The use of an inappropriate salt medium for rooting, despite lowering the salt concentration, results in a poor response (e.g. *D. tangutica*). *D. cneorum* is also dependent upon exogenous auxin to root, while the main effect of applying darkness to *cv. Aureo-marginata* during the root induction phase was to increase numbers of roots, but without significantly affecting the proportion of shoots rooting.

A pulse may also be used in vitro to induce root initials before transfer to compost and root emergence in a more conventional high-humidity propaga-

Table 6. Rhizogenesis in vitro using separate root induction and root expression phases (J. Kubba and T.R. Marks, unpubl.)

Species/cultivar	Root induction medium (7 days)	Root expression medium	Rooting percentage (LSD, $P = 0.05$)	Mean roots per rooted shoot (LSD, $P = 0.05$)
<i>D. cneorum</i>	1/2 LS	1/4 LS HF		
	0 μ M IBA		3	3.0
	100 μ M IBA		93 (11.2)	7.0 (1.92)
cv. Aureo-marginata	LS	1/2 LS HF		
	Light 100 μ M IBA		74	5.2
	IBA			
	Dark 100 μ M IBA		83 (24.1)	9.5 (2.78)
<i>D. tangutica</i>	WPM 100 μ M IBA	1/2 WPM HF	6	1.6
	LS 100 μ M IBA	1/2 LS HF	82 (8.4)	6.2 (1.05)

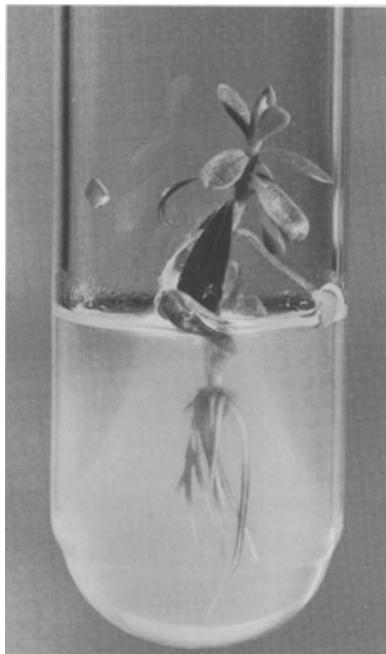


Fig. 6. *Daphne cneorum* shoot rooted in vitro. Shoot is from a 3.5-year-old culture

tion environment. Shoots (1.5 cm long) were exposed to different IBA concentrations on LS medium for 2 weeks, dipped in 0.1% w/v benomyl, and transferred to peat-based compost (Fisons Levington F1) in a high-humidity dry-fog environment. Both *D. x burkwoodii* and cv. Somerset rooted progres-

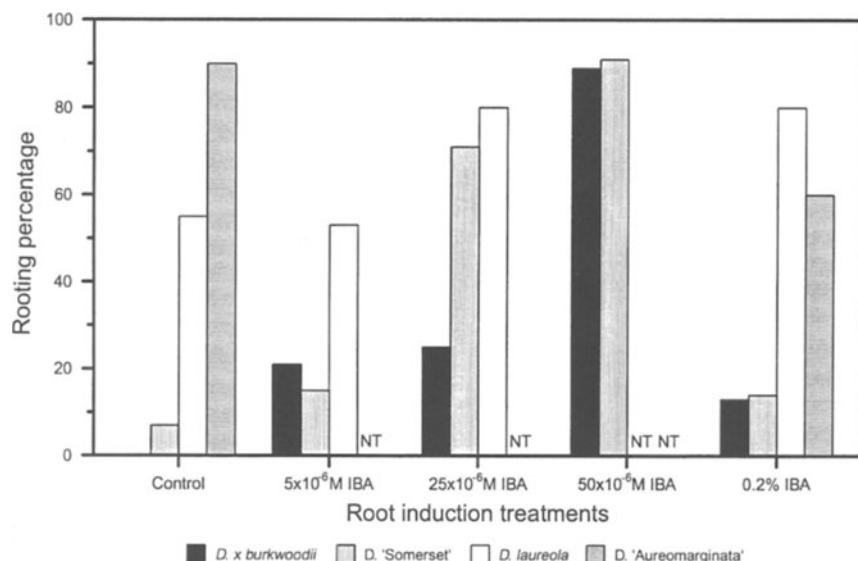


Fig. 7. Effect of different induction treatments upon root expression in compost of *Daphne* clones with different rooting characteristics. *NT* No treatment for missing species. Cultures are all 3 years old

sively better with increasing concentrations of IBA, but not when this was applied as a powder dip (0.2% w/w IBA in talc). Both *D. laureola* and cv. *Aureo-marginata* were easier to root and not dependent upon an exogenous auxin treatment, but were still responsive if auxin was applied in vitro, or as a powder during direct sticking into compost (Figs. 7 and 8). *D. x burkwoodii*, cv. *Somerset* and *D. cneorum* are similar in that they required either root induction, or additionally, root expression to take place in vitro; very few rooted following direct sticking.

Rooting by direct sticking (in vivo) is receiving more interest, especially in commercial operations, because of the advantages it has over in vitro methods (Debergh and Read 1991). For *Daphne* shoots, the propagation environment is paramount in determining successful rooting and weaning (Loach 1988). Table 7 summarises the results of using open mist or dry-fog environments on four in vitro-produced *Daphne* species with different rooting characteristics. In a subject such as *D. laureola*, which roots rapidly, differences were minimal between the environments. cv. *Aureo-marginata* was very sensitive to environment and shoots rooted only in the fog. *D. tangutica* (a different clone to that used in Table 6) was recalcitrant and produced only callus in both environments, whereas the related *D. retusa* rooted more readily, with more shoots rooting in the fog. To wean all *Daphne* plants successfully, the protocol described by Marks and Myers (1994) using two progressively less humid closed case environments after root development in fog was employed.



Fig. 8. *Daphne odora* cv. *Aureo-marginata* shoot rooted in vivo. Shoot is from a 2-year-old culture

Table 7. Direct (in vivo) rooting of shoots of evergreen *Daphne* species treated with 0.2% IBA w/w in talc in mist and fog environments (T.R. Marks, unpubl.)

Species/cultivar	Mist		Fog	
	Rooting (%)	No. of roots	Rooting (%)	No. of roots
<i>D. laureola</i>	83	4.3	92	3.9
cv. <i>Aureomarginata</i>	0	—	58	9.6
<i>D. retusa</i>	42	4.0	58	3.0
<i>D. tangutica</i>	0	—	0	—

LSD ($P = 0.05$): Rooting (%) = 27.9, No. of roots = 1.11.

The changes in physiology affecting root expression during micro-propagation can continue in the weaned and grown-on plant, such that subsequent conventional cuttings taken from in vitro-propagated source plants can show enhanced rooting potential. This has been demonstrated in plum (Howard et al. 1989), pear (Jones and Webster 1989) and rhododendron (Marks 1991). Plants of cv. *Aureo-marginata* were produced over a 3-year period, and cuttings 10 cm long were taken from plants 1, 2 and 3 years old ex vitro and from a conventional source plant. The leaves of these cuttings were dipped in 0.2% w/v benomyl and planted in a peat:grit/sand (3:1) propagation

Table 8. The effect of age after micropagation upon root expression in conventional cuttings taken from *Daphne odora* Aureo-marginata plants (T.R. Marks, unpubl.)

	1 year ex vitro	2 years ex vitro	3 years ex vitro	Conventional plant	LSD (<i>P</i> = 0.05)
Rooting (%)	100	100	100	100	—
No. of roots	29.0	20.7	18.2	11.8	4.5

bed in a wet fog environment (Agritec). No exogenous auxin was applied which might have masked differences in rooting potential. All shoots rooted, but the number of roots correlated inversely with the length of time after the micropagation event (Table 8). During the first 2 years ex vitro, growth of cv. Aureo-marginata shoots was vigorous with little branching, and flowering occurred only during the 3rd year. Similar success has been achieved with cuttings from 1-year-old plants of *D. laureola*. Enhanced rooting can be sustained in plants ex vitro (Howard et al. 1989), and offers an alternative means to exploit gains in rooting potential and cutting availability achieved through micropagation (Marks 1987).

4 Commercial Aspects

Within the UK there are three types of producers of micropropagated *Daphne* plants. These can be companies which are principally laboratory-based and sell directly to both small and wholesale nurseries. Alternatively, they are tissue-culture laboratories which are part of a larger nursery company, and either grow-on and sell the plants through conventional outlets of their parent company, or sell directly to others. The third group comprises commercial wings of teaching or research establishments, who generally deal with small specialist nurseries. Plants are principally sold through contracts with specific customers, or less frequently as part of the companies' general production range.

Current production of *Daphne* by micropagation is limited and few individual companies produce a sizeable quantity of plants in the UK. Where production is high, and this is still only of the order of 10000 plants p.a., it is principally of *D. odora*, notably cv. Aureo-marginata. Other species, such as *D. laureola* and *D. tangutica*, are produced in much lower numbers (e.g. 500–1000 p.a.). Elsewhere, such as in Australia, *D. odora* is also the principle species produced and accounts for 90% of *Daphne* sales. Other companies may micropagate a wider range of clones; for example one company produces 18 of those listed in Table 1, but because of limitations on commercial production can only currently produce ca. 2000 plants p.a. However, the production of relatively small numbers of *Daphne* has a significant niche within the UK market. The range of plants produced is dependent upon the ability of laboratories to overcome technical problems, while also being able to

produce saleable plants economically. This has probably limited the range to *D. odora* clones, where both in vitro factors and their requirements for cultivation have been well researched and worked through to a commercial scale.

Production is principally for home consumption, where *Daphne* are generally still regarded as specialist nursery or grower plants because of their problems of production and cultivation. Few, if any, are exported.

5 Conclusions

There is an increasing interest in *Daphne* as an ornamental plant, and micropropagation offers the most promising method for bringing slow-growing species into cultivation. Micropropagation also facilitates the establishment of virus-free nuclear stock, and may assist in the further exploitation of endogenous chemicals for pharmaceutical or pesticidal usage. However, tissue variability and sensitivity to PGRs illustrate that this is not an easy genus to culture.

6 Protocol

1. Use apical or distal axillary buds to ensure uniform culture development. Excise 2-cm apical sections.
2. Aseptically dissect or surface sterilize in sodium hypochlorite (0.7% w/v available chlorine) containing 0.05% Tween 20 and three washes in sterile deionised water. Plant vertically in medium.
3. Culture on Linsmaier and Skoog (1965) medium supplemented with 0.1–1 μ M BA, 6 mM hemicalcium gluconate (HCaG) and 87.6 mM sucrose at pH 5.2, and solidified with 5 g/l Agargel (Sigma). Shoot tips 1.5 cm long are subcultured every 4–6 weeks. Growth room conditions; 25 \pm 2 °C with 16-h photoperiod (light source and level as in Table 4).
4. For root induction in vitro, 1.5 cm shoots with active apices are placed on LS medium plus 30–100 μ M IBA and 6 mM HCaG. Transfer to hormone-free medium with reduced macroelements after 7 days.
5. For root induction in vivo; 1.5–2 cm shoots are treated with 0.1% w/v benomyl and bases dipped in 0.2% w/w IBA in talc containing 10% w/w captan before rooting in peat-based compost under high humidity dry fog (0.4 °C wet bulb depression).
6. In vitro or direct-rooted shoots are maintained in dry fog for 4 weeks, then weaned by progression through closed cases with >90% and 60–80% relative humidity, each for 7 days, with final hardening in a shaded glasshouse for 4 weeks.

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I.10 Micropropagation of *Dracaena* Species

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1 Introduction

1.1 Botany, Distribution, and Importance

The genus *Dracaena* (Agavaceae family) comprises about 50 species of woody stemmed plants in tropical regions of Africa and Asia (Bailey 1949), some of which are grown as ornamental plants. Similar and closely related to *Dracaena* are species of the genus *Cordyline*. Taxonomic position of some *Cordyline* species seems to be uncertain and conflicting, since they are also referred to as belonging to the genus *Dracaena*. Species recognized as ornamental plants include (Saakov 1983):

- D. fragrans* Ker-Gawl. (corn palm; tropical Africa). Green-foliaged plant and several distinct horticultural varieties/cultivars including: Massangeana, Lindenii, Rothiana, and Victoria.
- D. deremensis* Engler. (striped dracaena; tropical Africa) with varieties/cultivars Bausei and Warneckii,
- D. godseffiana* Sander. (gold dust dracaena; Congo)
- D. sanderiana* Sander. (ribbon plant; Congo)
- D. draco* L. (dragon tree; Canary Isles)
- D. arborea* (Willd.) Link. (East Africa and Angola)
- D. surculosa* Lindl. (Sierra Leone), variety/cultivar Punctata
- D. hookeriana* C. Koch (South Africa)
- D. reflexa* Lam. (Mauritius)
- D. goldieana* Baker (tropical South Africa)
- D. marginata* Lam (Madagascar dragon tree, silhouette plant; Madagascar), with variety/cultivar Tricolor
- D. indivisa* (G. Forst.) (cabbage tree; New Zealand) classified as *Cordyline indivisa* (G. Forst) Steud.
- D. congesta* Endl. (*D. stricta* Endl.; eastern Australia) classified as *Cordyline stricta* Endl.

Dracaena species are popular foliage plants of significant horticultural importance. Due to their subtropical origin, most of them must be grown indoors as potted plants. They can also grow under subdued light, which makes them a

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good choice for interior decoration of closed spaces in buildings like halls, offices, shops, restaurants, etc.

1.2 Conventional Propagation and Need for Micropropagation

Most *Dracaena* species are propagated vegetatively by stem cuttings. Some species, like *D. draco*, can be propagated by seeds. Small specimens can just be decapitated; the leafy top easily forms a root system, whilst the decapitated stem regenerates one or more flushes of leaves. Large-scale nursery production is based mostly on cuttings taken from mature plants grown outdoors in tropical plantations. The stem is cut into segments of variable length, which are prepared for shipment and sent to nurseries or directly to market. Once planted under favorable conditions, the apparently dry canes produce roots and leaves and in a short time reconstitute the whole plant, attaining a characteristic false palm appearance. Plants produced in this way are known as Ti plants. Species of *Yucca* and *Cordyline* can also be propagated in form of Ti plants. In some European countries, Ti plants of all three genera, and especially *D. fragrans*, are known under the common name “tree-of-life” plants.

Vegetative propagation of *Dracaena* species can be performed by various in vitro methods which all enable large scale commercial propagation. True micropropagation with axillary buds as starting explants is recommended especially for varieties with chimerical structure. Non chimerical species like green *D. fragrans* can be propagated by in vitro methods which employ callus in the early stages. The use of in vitro propagation techniques is far superior to conventional methods for many reasons, the most important of which is the tremendously increased potential for vegetative propagation. Large scale in vitro propagation is common practice in a number of nurseries, indicating the use of well elaborated but confidential propagation procedures.

2 In Vitro Culture Studies/Micropropagation

Studies on tissue culture and in vitro propagation of *Dracaena* species are summarized in Table 1. They cover more or less complete propagation procedures for four species; *D. deremensis*, *D. fragrans*, *D. godseffiana*, and *D. marginata*, and some hints and comments on the propagation of *D. congesta*, *D. pareyi*, *D. goldieana*, and *D. indivisa*.

Both reports presented by Debergh (1975, 1976) are studies centered on developing in vitro methods suitable for clonal propagation of various *Dracaena* species, especially those with chimerical structure. Although largely descriptive, they provide a scheme for micropropagation of *D. deremensis* cv. Warneckii and green *D. deremensis* and *D. fragrans*. Difficulties arising during propagation of chimerical cultivars of *D. deremensis* and *D. fragrans* are presented and discussed in detail.

Table 1. Studies on in vitro propagation of *Dracaena* species

Species	Primary explant	Plant growth regulators recommended for characteristic propagation stages (mg/l)			Reference
		Callus induction	Shoot multiplication	Pouting or pretransplant	
<i>D. deremensis</i>	Axillary buds	—	kin 5 NOA 1	IBA 2	Debergh (1975)
<i>D. fragrans</i>	Terminal shoot buds	—	kin 3 IAA 1	IBA 0.5	Debergh (1976)
<i>D. godseffiana</i> ^a			Ads 120		Miller and Murashige (1976)
<i>Dracaena</i> spp. ^b	Axillary buds	—	—	ex vitro	Debergh and Maene (1981)
<i>D. marginata</i>	Stem segments	2,4-D 0.5	kin 1	NAA 1	Chua et al. (1981)
<i>D. fragrans</i>	Various	2,4-D 10 ⁻⁶ M	—	—	Hunault (1979)
<i>D. fragrans</i>	Stem segments	2,4-D 0.25	BA 0.1 NAA 1	IBA 0.5	Vinterhalter (1989); Vinterhalter and Vinterhalter (1992)

Ads, adenine sulfate; NOA, naphthoxyacetic acid.

^a Procedure mentioned to be suitable for: *D. deremensis*, *D. goldieana*, and *D. marginata*.^b Species mentioned: *D. deremensis*, *D. congesta*, and *D. parreyi*.

Procedure for in vitro propagation of *D. godseffiana* (Miller and Murashige 1976) is part of a detailed study on micropropagation of four tropical ornamental plants, including also *Cordyline terminalis* Kunth, *Scindapsus aureus* Engler, and *Syngonium podophyllum* Schott. *D. godseffiana* is shrub-like and bears little similarity to other *Dracaena* species, which have a false palm appearance.

Results on tissue culture of *D. fragrans* Ker-Gawl. presented by Hunault (1979) are part of a much wider study which covers several members of the family Agavaceae, including also; *Sansevieria trifasciata* Prain, *S. cylindrica* Bojer, *Cordyline indivisa* Kunth, and *Agave* sp. In vitro propagation was not a primary target of this study, which is centered mostly on the investigation of organogenesis in leaf explants of *S. trifasciata* Prain. Results are well documented with histological investigations.

Debergh and Maene, (1981) presented a five-stage scheme for commercial propagation of ornamental plants by tissue-culture methods. The scheme was reported to be suitable for a variety of plant species including those from *Dracaena* and *Cordyline genera*. Report on in vitro propagation of *Dracaena marginata* cv. Tricolor by Chua et al. (1981) gives a procedure for rapid in vitro propagation via induction and differentiation of callus from stem explants.

Procedure for in vitro propagation of green-foliaged *D. fragrans* Ker-Gawl. (Vinterhalter 1989) is also based on the use of shoots regenerated from callus derived from stem explants. This procedure has been compared with those elaborated for in vitro propagation of *Cordyline terminalis* cv. Kiwi and *Sansevieria trifasciata* var. laurentii (Vinterhalter et al. 1989). The procedure has been further improved in studies in which *D. fragrans* was used as a model system to study factors which regulate formation, elongation, and branching of roots in vitro (Vinterhalter et al. 1990; Vinterhalter and Vinterhalter 1992).

2.1 Primary Explants and Culture Initiation

Primary explants for propagation of green and chimerical *D. deremensis* and *D. fragrans* (Debergh 1976), were 3-cm-long segments of the young stem containing four to five dormant lateral buds. In *D. godseffiana* (Miller and Murashige 1976) primary explants were shoot tips.

Primary explants for propagation of *D. marginata* (Chua et al. 1981) and *D. fragrans* (Vinterhalter 1989) were stem segments which were cultured on media supplemented with 2,4-D, to induce callus formation. The optimum 2,4-D concentrations for callus induction were 0.5 mg/l for the first and 0.25 mg/l for the second species. Callus of *D. marginata* differentiated two to seven shoots per culture on media with 1 mg/l kinetin. In *D. fragrans* the optimum shoot differentiation occurred if callus was subcultured on media with BA 0.5–1 mg/l and IBA 0.5–2 mg/l or NAA 0.1–0.2 mg/l (not shown in Table 1).

According to Hunault (1979), stem explants of *D. fragrans* cultured on media with 10^{-6} M 2,4-D produced a callus which was maintained on media with 2.5×10^{-6} M 2,4-D and 3×10^{-7} M BA as an undifferentiated tissue.

Shoots and roots differentiated on the callus upon decreasing 2,4-D concentration under 10^{-7} M (not shown in Table 1).

In all these reports, surface sterilization was performed with $\approx 0.5\%$ sodium hypochlorite prepared from commercial bleach. Also common to all reports was the use of MS (Murashige and Skoog 1962) macrosalts for media preparation.

In the scheme developed by Debergh and Maene (1981), special attention was paid to the initial propagation stage (stage 0). Donor plants maintained in the greenhouse at high temperature (25°C) and relatively low humidity (70%) were never watered overhead. The surface sterilization procedure recommended for *Dracaena* and *Cordyline* spp. was more elaborated. Material was immersed in 95% ethanol (30s), transferred to 0.5% HgCl_2 + a few drops of Tween 20 (3 min) and then twice treated with commercial bleach.

2.2 Shoot Cultures

Media used for shoot cultures of *D. deremensis* and *D. fragrans* (Debergh 1976) contained 5 mg/l kinetin and 0.1 mg/l NOA (naphtoxyacetic acid). A continuous production system could be established by reculturing the stump remaining after the excision of shoots which proliferated and elongated.

D. godseffiana (Miller and Murashige 1976) favored stationary liquid to agar-gelled or rotated liquid media. The recommended balance of plant growth regulators was 3 mg/l kinetin + 1 mg/l IAA. Supplements which promoted growth of cultures in multiplication stage were adenine sulfate 120 mg/l and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 170 mg/l. The increase of light intensity from 1000 to 3000 lx increased the number of shoots per culture more than twofold. Shoot multiplication was performed by enhancement of axillary branching. Long subculture duration lasting 7 weeks was necessary to enable newly formed shoot buds to elongate. Cultures were maintained for more than 2 years. Propagation rate was estimated at 300 000 plants per year.

For *D. marginata* (Chua et al. 1981) it is not clear whether a continuous propagation system via the use of shoot cultures has been established. It should be noted that 15% coconut milk was a standard media addendum in all propagation stages.

D. fragrans (Vinterhalter 1989) was maintained in the form of shoot clusters, groups of shoots growing from a common base (Fig. 1). Multiplication media contained 0.5–1 mg/l BA and 0.1–0.2 mg/l NAA. The shoot multiplication procedure was modified in later work (Vinterhalter et al. 1989, 1990), until a new formulation of plant growth regulators was achieved (Vinterhalter and Vinterhalter 1992) in which BA was decreased to 0.1 mg/l and NAA increased to 1 mg/l. This formulation completely prevented formation of callus on cultures, enabling a low but steady and predictable propagation rate. Average propagation rate was 1:1.6 expressed as the increase in the number of culture flask per subculture. Duration of subcultures was long, same as in *D. godseffiana*, exceeding 6 weeks. Separate shoot elongation stage was not necessary. The number of shoots ready for rooting (≈ 3 cm or longer) was 8–12 per culture flask, at the end of a subculture.



Fig. 1. Shoot clusters of *D. fragrans* on maintenance medium. 1 mg/l NAA and 0.1 mg/l BA

2.3 Rooting

In *D. deremensis* and *D. fragrans* (Debergh 1975, 1976), rooting was performed with 2 mg/l IBA using explants \approx 2 cm long. Rooting was 100% efficient.

Debergh and Maene (1981) recommended that, whenever possible, shoots should be transferred from in vitro conditions and rooted in vivo, i.e., they should be treated as ordinary cuttings. For *D. deremensis*, *D. congesta*, *D. pareyi*, and a number of other plant species, shoots were transferred to rockwool or other types of artificial substrates saturated with various root-promoting solutions among which standard solution contained 2 mg/l IBA. The practice of in vivo rooting has been readily and widely accepted for commercial propagation.

In the scheme for micropropagation of *D. godseffiana* (Miller and Murashige 1976), the rooting stage was replaced by a pretransplant stage. The idea was not only to induce formation of roots, but also to prepare plants for the transfer to in vivo conditions. Auxin, IAA was supplemented at 1 mg/l and light intensity was increased to 10000 lx. The pretransplant stage was not longer than 2 weeks, in order to prevent excessive root formation.

Shoots of *D. marginata* were separated from callus and rooted on medium with 1 mg/l NAA (Chua et al. 1981).

2.3.1 Rooting Experiments with *D. fragrans*

Formation, elongation, and branching of primary adventitious roots of *D. fragrans* in vitro has been thoroughly investigated in our laboratory (Vinterhalter 1989; Vinterhalter et al. 1990; Vinterhalter and Vinterhalter 1992). Major factors which were found to regulate rhizogenesis include: con-

centration of auxins, light intensity, macronutrient salts, carbohydrates, and initial length of shoot explants used for rooting.

In *D. fragrans*, as in most other plant species, auxins increased the number of roots per rooted culture and decreased the length of primary adventitious roots. The effect of IBA on basic rooting parameters is presented in Table 2. Optimal IBA concentration for rooting was considered to be 0.5 mg/l (Fig. 2), since for all parameters it enabled good but not highest values. If not otherwise stated, IBA at 0.5 mg/l was used in all subsequent rooting experiments.

Studies showed that shoots reaching the length required for rooting (≈ 30 mm) were photoautotrophic and that they efficiently utilize light available in the growth room for root formation. Root elongation was found to be directly dependent on irradiance, as can be seen from Fig. 3. In the absence of light (darkness), metabolism turned to dissimilation utilizing exogenously supplied sucrose (Fig. 4). Sucrose acted as a major factor, regulating root elonga-

Table 2. Effect of 0–2 mg/l IBA on standard rooting parameters. Results scored after 5 weeks. (Vinterhalter 1989)

IBA (mg/l)	Rooting (%)	Length of the longest root (mm) \pm SE	Roots per rooted shoot \pm SE
0.1	100	35.0 \pm 6.4	4.5 \pm 1.8
0.2	100	30.2 \pm 6.8	4.5 \pm 1.9
0.5	100	20.9 \pm 5.3	5.6 \pm 1.8
1	100	11.3 \pm 3.9	5.8 \pm 1.6
2.0	100	7.6 \pm 1.8	8.7 \pm 2.6

Fig. 2. Rooted plantlets of *D. fragrans*. 0.5 mg/l IBA, duration of rooting 5 weeks



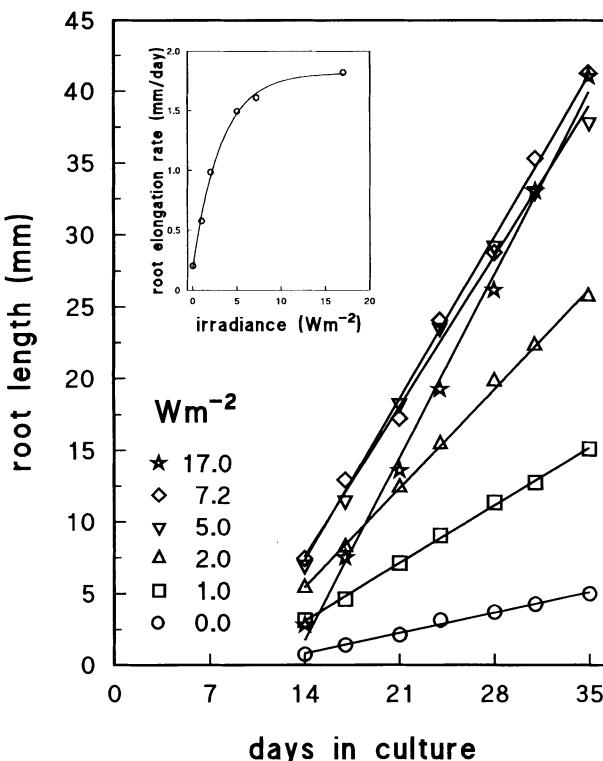


Fig. 3. Effect of irradiance on root elongation. Insert presents root elongation rate (mm/day) plotted against irradiance (W/m^2). (Vinterhalter et al. 1990)

tion only in darkness, but it could not completely substitute for the absence of light. The lowest length in light obtained on sucrose-free medium was still higher than maximum root length in darkness registered at 8% sucrose (Fig. 4, insert). The effect of sucrose on root elongation in light was less pronounced than in darkness and the maximum was at 3% sucrose. It is interesting to note the marked difference in maxima for sucrose-regulated root elongation in light and darkness. The requirement for high light intensities and high carbohydrate concentrations in the medium have also been reported for *D. godseffiana* (Miller and Murashige 1976). One of the side effects of photoautotrophic growth of *D. fragrans* cultures was that the length of initial shoot explants also affected subsequent root elongation. Studies with shoots ranging from 20–60 mm in length showed that root elongation increased with shoot length, reaching a maximum in shoots 47–48 mm long (Fig. 5).

Root elongation and lateral root formation were strongly affected by macronutrient salts. At full-strength MS macrosalts, the growth of primary adventitious roots was rectilinear, meaning that roots elongated straight down through the medium. The decrease of concentration of macrosalts in the medium did not affect the total (absolute) root length. It actually induced curving of roots, significantly decreasing their relative length, i.e., the depth of root penetration through the medium (Table 3).

Fig. 4. Effect of sucrose on root elongation in light and darkness. *Insert* shows root elongation rate (mm/day) plotted against sucrose concentration

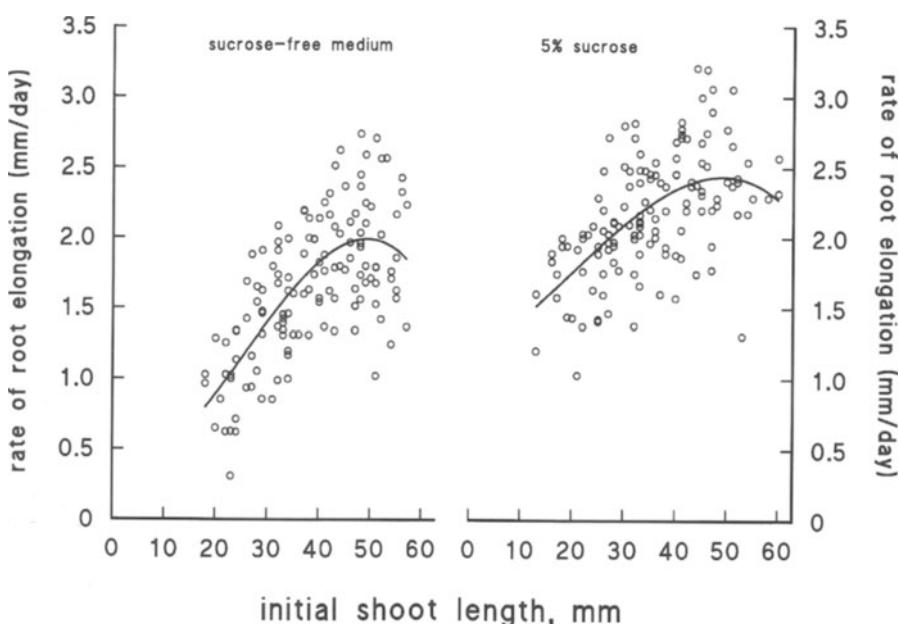
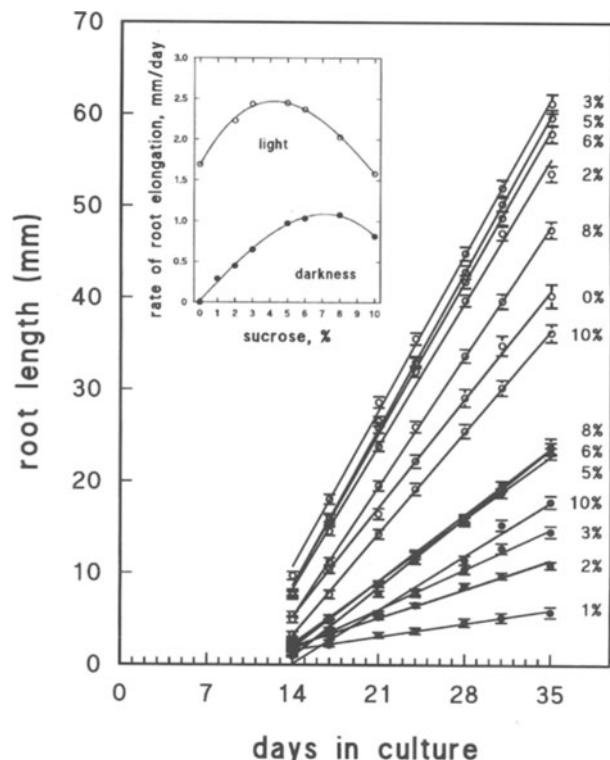


Fig. 5. Effect of initial shoot length on the root elongation rate on sucrose-free and medium with 5% sucrose. 130 shoots of various length (20–60 mm) were excised from a single culture flask. Each point in the graph corresponds to the root elongation rate of a single explant

Table 3. Effect of inorganic nutrition on root length, RCI index, rooting percentage, and formation of lateral roots. Results scored after 5 weeks

Inorganic salts present in the medium	No. of shoots	Relative root length (mm) ± SE	RCI index (%) ^a	Roots per shoot (rooted) ± SE	Percentage of roots forming lateral roots	Lateral roots per shoot ± SE
Complete MS (macro + micro + iron)	76	54.9 1.0	92.1	4.4 0.2	5.7	0.7 0.2
1/1 macrosalts	169	57.1 1.0	91.8	4.0 0.1	5.5	0.7 0.2
1/2 macrosalts	79	55.5 1.0	89.1	3.7 0.2	12.6	1.9 0.5
1/3 macrosalts	78	49.8 1.2	79.3	3.6 0.2	32.7	4.8 0.6
1/5 macrosalts	78	38.1 1.0	64.7	3.3 0.1	70.9	8.2 0.6
1/10 macrosalts	76	29.4 1.3	52.9	3.2 0.1	86.4	14.0 0.7
Micronutrient salts	79	31.8 1.6	60.4	3.1 0.1	67.5	13.4 1.2
MS Iron-complex	96	32.4 1.9	59.1	3.4 0.1	62.1	11.9 1.0
None (salt-free)	100	26.1 1.3	58.5	3.1 0.1	83.4	19.6 1.2
1/1 macrosalts, $MgSO_4$ omitted	83	51.0 1.0	94.4	4.3 0.1	67.5	13.7 1.1
AMS ^b macrosalts, K_2SO_4 omitted	99	41.5 1.1	92.3	4.1 0.1	61.1	8.0 0.7
AMS macrosalts, $MgCl_2$ omitted	99	51.9 1.0	91.6	4.1 0.1	2.0	0.3 0.1
AMS macrosalts with 0.5 mM K_2SO_4	99	50.3 0.9	94.1	4.1 0.1	2.0	0.3 0.1
AMS macrosalts + MS iron, with K_2SO_4 omitted	100	50.9 0.8	95.1	4.2 0.2	4.1	0.5 0.2

^aRCI index is parameter of root curving calculated as relative/absolute root length $\times 100$ (Vinterhalter and Vinterhalter 1992).

^b AMS – Alternative MS macrosalt formulation prepared from seven macronutrient salts has the same ionic strength as in MS (Vinterhalter and Vinterhalter 1992).

Fig. 6. Abundant lateral root formation on shoots rooted on media with 1/10 MS macrosalts. (Vinterhalter and Vinterhalter 1992)

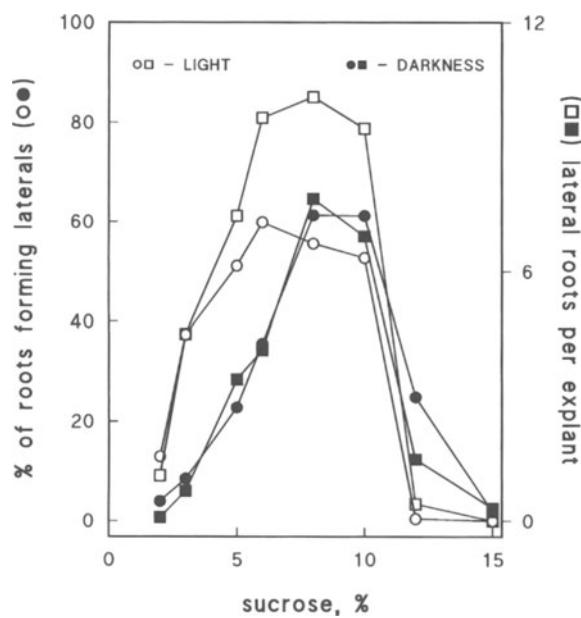


Fig. 7. Effect of sucrose concentration on the formation of lateral roots

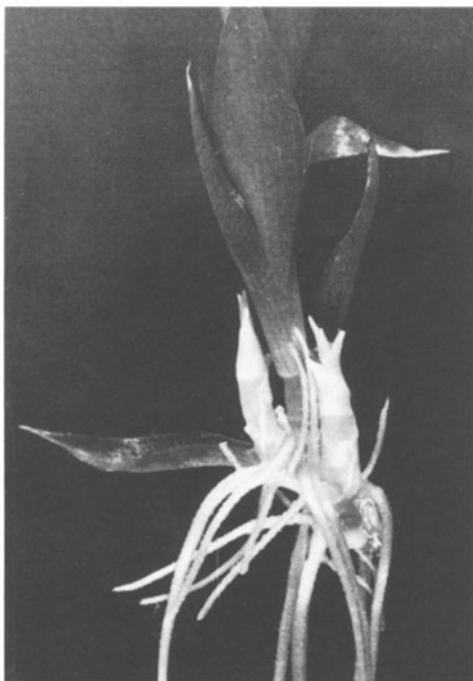


Fig. 8. Formation of etiolated axillary shoot buds on shoots rooted in darkness on media supplemented with 5% sucrose

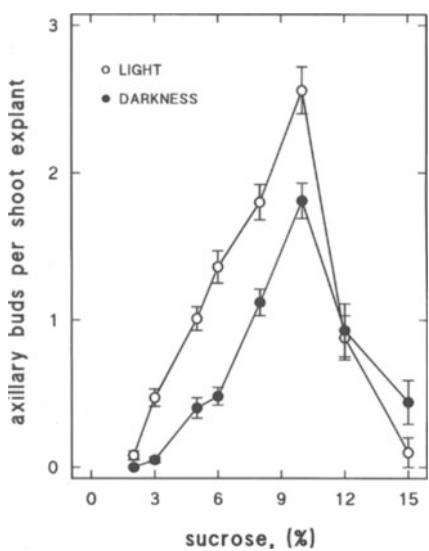


Fig. 9. Effect of sucrose on the formation of axillary buds during in vitro rooting

In media supplemented with MS inorganic salts at full strength, the formation of lateral roots was almost completely inhibited and appeared in no more than 5% of all primary adventitious roots. This inhibition was found to result from improper balance of the two nitrogenous ions (NH_4^+ and NO_3^-) and SO_4^{2-} ion (Vinterhalter and Vinterhalter 1992). Inhibition of lateral root formation could be overcome by decreasing the concentration of either one or both groups of ions (Table 3 and Fig. 6).

Further investigations showed that lateral root formation is regulated also by carbohydrates (Fig. 7). In both light and darkness, lateral root formation increased with sucrose, reaching maximum at 8% sucrose. Thus the increase of sucrose concentration in the medium from standard 2% enabled lateral rooting even in the presence of full-strength MS inorganic salts. In this case, light could not compensate for the absence of sucrose.

Sucrose had an additional morphogenetic effect during rooting; it stimulated formation of true axillary shoot buds side by side with roots. Formation of axillary buds (Fig. 8) was directly related to concentration of sucrose (Fig. 9) and increased linearly reaching maximum at 10% sucrose in both light and darkness. Axillary buds in darkness were etiolated. These axillary buds strongly resembled tiller buds of grasses.

2.4 Adaptation of Rooted Plantlets

Rooted plantlets of *D. deremensis* (Debergh 1975) were planted in peat and further grown under intermittent mist. Survival was 100% and growth was normal.

In the propagation scheme devised by Debergh and Maene (1981), rooting and adaptation of rooted plantlets were performed as a single stage. The adaptation was performed in plastic tunnels in winter and under intermittent mist in summer.

Rooted plantlets of *D. godseffiana* (Miller and Murashige 1976) and *D. marginata* (Chua et al. 1981) were planted in substrate composed of equal parts of peat, vermiculite, and perlite. Adaptation was performed in a greenhouse, applying intermittent mist. Plants of *D. godseffiana* were shaded with Saran wrap to provide 60% shade.

Rooted plantlets of *D. fragrans* (Vinterhalter 1989) were easily adapted in the greenhouse using various substrates based on peat. Use of intermittent misting was not required to obtain nearly 100% adaptation.

2.5 Phenotypic Stability

In both *D. marginata* and *D. fragrans*, the use of callus at early stages of propagation did not affect the clonal characteristics of propagated plants. In *D. fragrans*, stability of the main clone has been routinely checked for more than 10 years, and in this period off-type plants were registered only twice, each time in a single culture vessel. Thus, *D. fragrans* can be considered as a

species of remarkable stability during in vitro propagation. A propagation procedure similar to the one used for *D. fragrans* enabled establishment of a stable clone in *Aechmea fasciata* Baker (Vinterhalter and Vinterhalter 1994), a species known for its instability under conditions of in vitro culture (Jones and Murashige 1974; Zimmer and Pieper 1976).

The two aberrant *D. fragrans* shoot clusters proliferated mostly shoots with randomly variegated leaves and occasionally white or yellowish albinos which could not be further maintained in in vitro culture. Attempts to propagate variegated plants continuously were not successful, since they were steadily outbred in favor of albinos.

In *D. godseffiana* no obvious morphological deviations were encountered in multiplied shoots and propagated plants (Miller and Murashige 1976) although numerous subcultures were performed over a period lasting more than 2 years.

The propagation procedure for chimerical varieties of *D. deremensis* and *D. fragrans* presented by Debergh (1976) was successful if stem segments with lateral shoot buds were used as explants. Other types of primary explants were suitable only for plants of green phenotype, since chimerical varieties often produced off-type (aberrant) plants. The basic problem which was encountered with chimerical plants was that shoots of obviously different phenotypes could develop on the same shoot cluster, making the procedure unsuitable for commercial exploitation.

3 Summary and Conclusions

Dracaena species are good example of plants in which clonal propagation can be performed by various in vitro methods. However, micropropagation is strongly recommended, especially for varieties with chimerical structure. In a number of species micropropagation procedure can be shortened, performing the rooting stage ex vitro.

In our studies on in vitro rhizogenesis of *Dracaena fragrans*, formation and elongation of primary adventitious roots could be modified by a number of factors including auxins, light intensity, and mineral and carbohydrate nutrition. Moreover, inorganic and carbohydrate nutrition had a distinct morphogenetic effect, since they regulated formation of lateral roots and axillary buds.

4 Protocol for In Vitro Propagation of *D. fragrans* Ker-Gawl

Starting material: use healthy specimens grown in the greenhouse

Preparation of material: remove all leaves, and cut young portions of stem into 5–6-cm-long segments

Surface sterilization: 20 min in 10% commercial bleach (4–5% NaOCl) + one to two drops of some liquid detergent

Primary explants: thoroughly rinse the stem segments in autoclaved water. Cut them aseptically into 3–4-mm-thick segments and place on the medium for callus induction

Media composition and preparation: basal medium (BM) contains Murashige and Skoog (1962) inorganic salts, vitamins, and inositol, with 2.0–3.0% sucrose and 0.64–0.7% agar. Adjust pH to 5.8 prior to autoclaving, which should be performed for 20–25 min at 114–115 °C.

Callus induction medium: BM + 0.25 mg/l 2,4-D

Shoot differentiation medium: BM + 0.5–1 mg/l BA + 0.1–0.2 mg/l NAA

Contamination screening: shoots selected for establishment of stock cultures should be screened for presence of microorganism on a broad spectrum media (agar + peptone + yeast extract).

Shoot multiplication and elongation: BM 0.1 mg/l BA + 1 mg/l NAA

Rooting: use shoots \approx 30 mm long and media with 0.5 mg/l IBA

Temperature in the growth room: 25 \pm 2 °C

Photoperiod: 16/8 h light to darkness

Light source: cool white fluorescent lamps

Standard irradiance: 33.5–46.5 $\mu\text{mol/m}^2/\text{s}$ at the culture level (7.2–10 W/m²)

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I.11 Micropropagation of *Gerbera*

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1 General Account

1.1 Botanical Characteristics and Importance

The genus *Gerbera* (Compositae family), covers around 30 herbaceous and perennial species originating from southern and eastern Africa, South America, and Asia (Nepal, Manchuria). The plants remain vegetative until the initiation of a rosette of 7–26 leaves, then become reproductive and produce capitula. Growth is sympodial, progressing by the reactivation of the axillary bud located at the axil of the penultimate leaf; this bud initiates three leaves before turning reproductive (Leffring 1978).

Most of the species with colored capitula of medium size are allogamous (*G. jamesonii*, Fig. 1a, *G. viridifolia*, *G. aurantiaca*, *G. kraussii*). The other species (*G. kunzeana*, *G. cordata*, *G. nivea*, *G. piloselloides*, Fig. 1b) are autogamous, with more or less cleistogamous flowers.

The commercial varieties are derived mainly from *G. jamesonii*, described for the first time in 1889 (Codd 1979), and should be designated *G. jamesonii hybrida* or *G. hybrida*.

The production of *Gerbera* for cut flowers was initiated at the beginning of the 20th century in France. The cultivated area in Europe increased from 140 ha in 1975 to 600 ha in 1986, the main producing countries in 1988 being The Netherlands (248 ha), Italy (120 ha), Germany (70 ha), and France (30 ha). In 1992, the surfaces were reduced to 400 ha, with half in The Netherlands (Verdegaal 1992). Outside Europe, recent estimations reported production from Japan (30 ha) and Israel (20 ha). Increasing surfaces are also devoted to *Gerbera* in Korea and Taiwan (50 ha in 1994).

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Fig. 1. **a** *Gerbera hybrida* Bol. L., 6 months after acclimatization. Bar 10cm. **b** *Gerbera piloselloides* (L.) Cass., 6 months after acclimatization. Bar 5cm. **c** *Gerbera hybrida* Bol. L.: shoot cluster after 1 month on multiplication medium. Bar 1cm. **d** *Gerbera piloselloides* (L.) Cass.: shoot cluster after 1 month on multiplication medium. Bar = 1cm

1.2 Genetic Improvement

The large diversity of phenotypes among the gerberas derived from *G. jamesonii* could result from the combined effects of cultural background distant from the natural habitat, strong selection pressure based on esthetic criteria only and, probably, interspecific crosses (in particular with *G. viridifolia*).

G. jamesonii is diploid ($2n = 2x = 50$). Meiosis of haploid plants regenerated in our laboratory from unfertilized ovules never exhibited chromosome pairing. Surprisingly, although chromosome numbers were $2n = 50$ for *G. kunzeana* (Rongeot 1989), *G. viridifolia*, and *G. piloselloides* (Reynoird et al. 1993), these two last species displayed a 1.5-fold increase in DNA content per nucleus in contrast with *G. hybrida*.

Genetic studies revealed a small number of Mendelian characters: the presence of cyanidin in the pappus ("dark heart" character) is monofactorial; the production of pelargonidin and kaempferol is dominant over cyanidin and quercetin (Tyrach 1993); the anthocyanin glycosylation in positions 3 and 5 is under the control of two dominant and complementary genes (J. Meynet, unpubl.). However, most of the characters interesting for agronomy or esthetics are polygenic and their strong heritability allows efficient selection (de Jong and Garretsen 1985; Drenman et al. 1986). Some morphological traits such as diameters of capitulum heart and stem, or width and number of ligules, are strongly bound and are involved in the definition of the phenotypes (De Leo and Ottaviano 1978).

Gerbera is extremely susceptible to the depressive effects of consanguinity. Hybridization between distant parents provided with a strong heterozygosity and complementary characters has resulted in offspring sufficiently diversified to insure the appearance of new cultures and fast, continuous genetic progress.

For cut flowers, some of the most interesting characters, i.e., size of the capitula (Drenman et al. 1986), branching and productivity (de Jong and Garretsen 1985), vase life (Meeteren 1978; de Jong and Garretsen, 1985; Dubuc et al. 1985; de Jong 1986), and flowering precocity (J. Meynet, unpubl.) display a strong additive heredity. The other characters are color (Barigozzi and Quagliotti 1978), resistance to pathogens, seed production (Schiva 1978) and suitability for outdoor culture.

1.3 Conventional Practices for Propagation

Gerbera was multiplied exclusively through the sexual pathway until 1971. An efficient method of vegetative multiplication through cutting by division of the crown was established (Leffring 1971), the multiplication rate varying between eight and ten per year. Kaminek et al. (1987) recorded a threefold increase rate following cytokinin sprays.

Limits and respective disadvantages of these methods were compared by Duclos and Meynet (1977). Bulk selection leads to heterogeneous seeds from

which it is possible to select lines with better performance than the initial population. In contrast, multiplication through cutting gives rise to early flowering and less ramified plants and is, consequently, less productive than plants obtained from seeds. The low multiplication rate via vegetative multiplication leads to long delays for commercial distribution, heavy manufacturing costs, and inertia in variety management, which are commercial risks. Using mother plants after 1 year of flower production has had serious consequences on the sanitary state of the young plants, namely by propagation of wilt diseases due to vascular fungi (*Phytophthora cryptogea*). For such reasons, both these conventional practices have now been relinquished in favor of in vitro micropropagation.

1.4 Biotechnological Requirements

1.4.1 Plant Production

The search for efficient protocols of vegetative propagation was made necessary owing to the 15–20 million plants required each year to satisfy the European market. The evolution of biotechnological methods was stimulated by commercial demand for rapid, large-scale and true-to-type multiplication. An important factor has been the necessity to establish virus-free plants (i.e., cucumber mosaic virus and tobacco rattle virus), also uncontaminated by *Phytophthora cryptogea* Pethybr. and Laff (Maia et al. 1983). These techniques of production take full advantage of a range of artificial and inert substrates.

1.4.2 Plant Breeding

The main challenge for biotechnology in this field is to contribute to the enlargement of genetic variability in *Gerbera*.

1. *Resistance to pathogens*: due to the increased availability of pathogen-free varieties in in vitro cultures, and the use of inert substrates, resistance to *Phytophthora*, *Verticillium*, *Fusarium*, and *Rhizoctonia* is presently of reduced priority. However, *Botrytis cinerea* and pests (white flies, leaf miners, and thrips) still present real problems.

2. *Ability to vegetative propagation*: the capacity of species to be propagated through in vitro cultures, including somatic embryogenesis, is highly variable, and represents an area where great improvement is needed.

3. *Morphological diversity*: biotechnology could be used to overcome interspecific limits and to extend the variability towards some new characters (blue or mauve and true violet colors, strong fragrance, tubular ligules, limitation of foliage development, etc.). Limitation of the instability in anthocyanin contents caused occasionally by seasonal variations in bicolored capitula, and the loss of color intensity under sunny conditions in pink cultivars, could be useful.

2 In Vitro Studies and Micropagation

2.1 Commercial Micropagation via Direct or Indirect Axillary Bud Development

2.1.1 Establishment

Direct in vitro establishment was performed initially from *G. hybrida* shoot tips by Murashige et al. (1974). A two-step disinfection protocol was developed to prevent very high contamination rates due to pilosity. Huang and Chu (1985), peeling off the periderm of the rhizome located beneath the rosette, obtained better rates with a single one-step protocol. In our technique (for details see Sect. 4), applied on a large commercial scale, we flamed the shoot tips briefly several times and thus obtained 30% clean cultures, avoiding classical disinfection.

Methods of indirect establishment were also developed using excised capitula or the upper part of the flower stem from which vegetative buds were obtained, then subsequently micropagated via axillary branching (Table 1). This was advantageous due to the preservation of the mother plant, and limited the extent of contamination. Except in Pierik et al. (1973), the cultures were placed usually under a 16-h daily illumination fluorescent light.

Pierik et al. (1973) initiated multiplication from fully developed capitula. Pieces of capitula were first placed under darkness at 25°C for 4 weeks, then transferred under continuous fluorescent light (2100lx) at 23°C for another 4-week period, on a basal medium with 10mg/l BA. For all the tested cultivars except the yellow ones, each explant gave rise to two to three vegetative buds, which were subsequently multiplied.

Another method of establishment was developed by Maia et al. (1983) using involucral bracts from immature capitula. The excised capitula were flamed briefly then individual bracts were placed on an MS-derived medium with 0.5mg/l IAA and 0.5% agar. Laliberté et al. (1985) used capitula split into 20 pieces, which regenerated approximately 20 buds each after 12 weeks on medium containing 1 or 2mg/l BA and 0.1mg/l IAA. Chu and Huang (1983) initiated bud regeneration from the upper part of stem after 16 weeks on MS-derived medium with 10mg/l BA.

Although genotype effects are important, different regions of the inflorescence appear to be able to give rise to buds. There is a lack of information on both the origins of the buds regenerated from capitula (adventitious formation from receptacular tissues or reorientation of premeristematic tissues) and the risks of genetic variation through a possible indirect regeneration.

2.1.2 Mass Propagation

Whatever the establishment method, the mass propagation phase is always achieved via axillary bud development (Fig. 1c,d) on media derived from those

Table 1. Summary of micropropagation studies conducted on *Gerbera* species

<i>Gerbera</i> species/cv.	Explant used	Initial multiplication rate	Phenotypic conformity	Reference
<i>jamesonii</i> hyb. 1 red clone	Midrib from just-developing leaves on greenhouse plants	0; only callus and root induction	NR (not reported)	Pierik and Segers (1973)
<i>jam.</i> hyb. 1 red clone	Receptacle from fully developed capitula on greenhouse plants	1–2 in 2.5 months	NR	Pierik et al. (1973)
<i>jam.</i> hyb. 25 clones	Shoot tip from greenhouse plants	6 in 1 month	Yes	Pierik et al. (1975)
<i>jam.</i> hyb. cv. Vulkan	Intact leaves on in vitro shoot clusters	Only incidental	NR	Murashige et al. (1974)
<i>jam.</i> hyb. cv. Arendsoog, Beatrix, Continent, Supergiant	Upper part of flower stem with developed capitulum	5–6 in 4 months for yellow cv. only	NR	Hedrich (1979)
<i>jam.</i> hyb. 30 cvs	Immature capitula	15–30 buds in 1.5 months	Yes	Chu and Huang (1983)
<i>jam.</i> hyb. cv. Pastourelle, Mardi gras	Immature capitula	80–300 buds in 3 months	NR	Maia et al. (1983)
<i>jam.</i> hyb. 27 cvs	Leaves from in vitro shoot clusters	8–11 in one months	NR	Laliberté et al. (1985)
<i>jam.</i> hyb. cv. Petra, Clementine, 616, 11 clones	In vitro shoot apices, petioles	Incidental; only from petioles	NR	Jerzy and Lubomski (1991)
<i>jam.</i> hyb. cv. Terracina	Petioles from in vitro shoot clusters	Buds on 33% of explants in 2 months	NR	Ruffoni and Massabò (1991)
<i>jam.</i> hyb. 2 clones <i>piloselloides</i> L. Cass. <i>viridifolia</i> Schultz Bip.	Developing leaves from in vitro shoot clusters	3–6 buds on 50–90% of explants in 2 months	Yes	Elomaa et al. (1993)
				Reynoard et al. (1993, 1994)

used for establishment, but with lower concentrations of cytokinins. Murashige et al. (1974) used a modified MS (Murashige and Skoog 1962) medium with additional phosphate, adenine sulfate, and high sucrose concentration. High concentration of kinetin (10mg/l) was necessary to increase the coefficient of multiplication. Low IAA concentration (0.5mg/l) improved the vigor of the cultures. The light intensity was crucial for the multiplication rates, and optimum results were obtained with 1000lx. The photoperiod requirements ranged from 12 to 16h daily for the 1000lx intensity. Requirements for higher light intensities associated with low sucrose concentrations were reported by other authors. Maia et al. (1983) used a first medium differing from the establishment medium by lower hormone concentrations (0.2mg/l IAA and 1mg/l BA), then the subsequent subcultures for mass propagation were carried out on a second medium with doubled IAA and BA concentrations. Huang and Chu (1985) reported that high BA concentrations (5mg/l) improved the multiplication rate. Laliberté et al. (1985) observed that the differences between clones for cytokinin requirements were more important in the subsequent transfers than in the first subcultures.

Although the yield of in vitro multiplication was shown to increase up to 9 weeks (Murashige et al. 1974), a 4–5-week passage period was generally chosen as standard. The monthly multiplication rates varied between 4 to 7, with strong differences among clones.

2.1.3 Rooting and Acclimatization

Pierik et al. (1975) and Laliberté et al. (1985) noted a strong effect of shoot sizes on rooting efficiency. To prepare the shoots for rooting, Maia et al. (1983) increased the duration of the last subculture on the multiplication medium and lowered the hormonal concentrations by half. Then the shoots were individually transferred to a rooting medium with 2.5mg/l IAA at 23°C under the same conditions of illumination. For Murashige et al. (1974), highest rooting efficiency was obtained with highest IAA levels (up to 10mg/l). The main impact was on the number of roots per shoot. Light intensities higher than 1000lx increased the percentage of rooted shoots when the cultures were exposed to 16h illumination daily. In contrast, efficient rooting was obtained by Pierik et al. (1975) on modified MS medium added with 10mg/l IAA under low light intensity (800lx). IBA was also found to induce shorter roots than IAA. The first roots appeared after 10 days and the shoots could be acclimatized immediately (Murashige et al. 1974).

For Huang and Chu (1985), root induction could be achieved by dipping the shoots in 0.1% IBA solution for 30s before direct transplanting in soil or inert substrates. In our experience, root induction was conducted after a last propagation subculture of 6 weeks on multiplication medium in order to obtain sufficient shoot elongation. Strong differences in rooting ability were observed among the genotypes revealing interactions between clone and medium, namely for the dwarf varieties propagated for potted plants.

2.1.4 Phenotypic Conformity

After acclimatization, most of the rooted plantlets survived well without displaying any phenotype variation. Only some plants with bushy phenotypes resulting from excessive branching were observed occasionally. These plants were often malformed and displayed a reduced ability for acclimatization, as well as flowering delay and abnormal capitula. Differential effects of in vitro culture corresponded to the clonal capacity to recover an ex vitro apical dominance until flowering (Meynet 1983). Excessive branching increased with successive subcultures, especially when high hormone concentrations were used. The extension of the rooting period and a hormone concentration decrease allowed reducing this excessive branching, but it made a repeat of the cultures necessary. Temporary abnormalities had to be dissociated from stable ones which remained after traditional cutting and corresponded to multiplication untrue-to-type (Meynet 1983), probably bound to a dedifferentiation phase.

Using shoot tips as initial explants reduced this risk. Only Murashige et al. (1974) mentioned histological observations and their chromosome counting for 25 propagated clones revealed no chromosome variation when new shoots arose as outgrowths of axillary buds without callus formation.

2.2 Adventitious Bud Regeneration from Leaves

Although commercial production of *Gerbera* has never been achieved through regeneration of adventitious buds from leaves, this approach could represent an efficient alternative to the current methods, provided that the phenotype and flower production of the regenerants be maintained. Furthermore, after mutagen treatment, the chances of obtaining stable mutants from adventitious shoots were far greater than from axillary shoots.

Experiments on leaf explants excised from mature plants and cultured under darkness at 25–29 °C resulted in root and callus formation (Pierik and Segers 1973). Only incidental bud regeneration from intact leaves of in vitro plants of one cultivar was described on a modified MS medium supplemented with 1 mg/l BA and 0.1 mg/l GA₃ at 22 °C (Hedtrich 1979). Jerzy and Lubomski (1991) observed bud regeneration at the base of leaf petioles from shoot clusters on a modified MS medium with BA or kinetin at 24 °C after 6 weeks, BA being more efficient than kinetin. Strong genotype effects were reported and cytokinin concentration effects were not significant. Four to six shoots were regenerated per leaf under the best conditions. Additional research indicated that in vitro plant leaf petioles and shoot apices were able to regenerate some buds, whereas leaf lamina were able to regenerate only roots and callus (Ruffoni and Massabo 1991). More recently, Elomaa et al. (1993) reported another method of regeneration from pieces of petioles on MS basal medium with 0.1 mg/l IAA, 1 mg/l BA, and 1 mg/l zeatin. In most of the experiments from leaves of in vitro plants (Table 1), the rates of bud regeneration remained very low and no information was available about conformity.

In our experience, bud regeneration was achieved from leaves of in vitro shoot clusters for two clones of *G. hybrida* and for the five wild types *G. viridifolia*, *G. piloselloides* (Reynoird et al. 1993; 1994), *G. adriana*, *G. nivea*, and *G. cordata* (J.P. Reynoird, unpubl.). Great difference was observed in morphogenetic capacities between the developing and the fully expanded leaves and a strong clonal effect (Table 2). For a reactive clone of *G. hybrida*, up to 90% regeneration could occur from young leaves (Fig. 2a), but the fully expanded leaves (Fig. 2b) regenerated only when the medium was supplemented with TDZ (Table 3). For a recalcitrant clone, only 50% of the young leaves regenerated buds and regeneration was greatly enhanced by addition of auxin transport inhibitor TIBA (Reynoird, 1996). Bud regeneration occurred usually after a phase of callogenesis.

G. viridifolia exhibited reaction similar to *G. hybrida*. Addition of TDZ to the basal medium enhanced the regeneration for the recalcitrant *G. piloselloides* (Table 4); but for these two wild species, only the developing leaves were reactive. Regenerants of *G. hybrida*, *G. viridifolia*, and *G. piloselloides* were cultured until flowering and displayed no phenotypic variations (Table 5).

The stability of the ploidy levels of the regenerants was controlled on the basis of flow cytometric data and by chromosome counting in root apices (Reynoird et al. 1993). The chromosome number was $2n = 50$ for all the genotypes (Fig. 2c). Shoot apices and in vitro or in vivo leaves were composed mainly of 2C cells, and the DNA content per nucleus was stable in both

Table 2. Effect of leaf development and BA concentration on formation of adventitious buds for *G. hybrida* clones 10 and 11 leaves regenerating buds (%) and mean number of buds per reactive leaf ()

Leaf development	Clone 10			Clone 11		
	BA 2.5 μ M	10 μ M	25 μ M	BA 2.5 μ M	10 μ M	25 μ M
DL	83.8 (2.1)	86.5 (2.6)	97.8 (2.0)	36.6 (2.1)	49.4 (3.2)	47.1 (3.7)
EL	0	0	0	0	0	0

DL: developing leaf (length <2 mm); EL: fully expanded leaf (length >6 mm).

Table 3. Effect of addition of thidiazuron to 10 μ M BA on the percentage of *G. hybrida* clones 10 and 11 explants provided with buds and on the mean number of buds per explant ()

Leaf development	<i>G. jamesonii</i> Clone 10			Clone 11				
	TDZ (μ M)	0	0.05	0.5	TDZ (μ M)	0	0.05	0.5
DL	86.5 (2.6)	76.1 (2.9)	70.5 (4.4)	49.4 (3.2)	38.1 (3.7)	39.2 (3.0)		
EL	0	38.4 (4.7)	40.0 (4.3)	0	0	0		

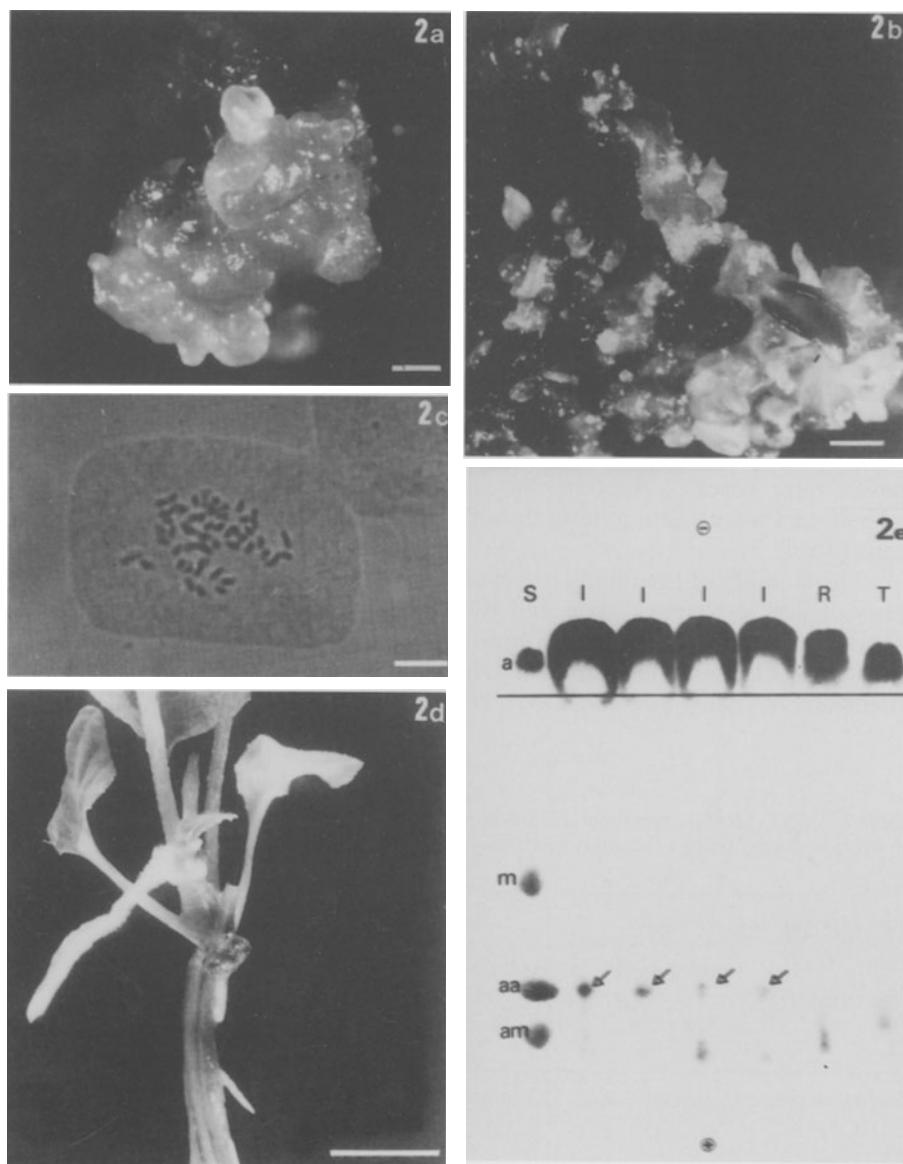


Fig. 2. **a** Behaviour of *G. hybrida* juvenile leaf cultured for 1 month on bud regeneration medium containing $10 \mu\text{M}$ BA and $2.5 \mu\text{M}$ NAA. Bar 2 mm. **b** Behavior of *G. hybrida* fully differentiated leaf cultured 2 months on bud regeneration medium containing $10 \mu\text{M}$ BA, $2.5 \mu\text{M}$ NAA, and $0.5 \mu\text{M}$ TDZ. Bar 2 mm. **c** Feulgen-stained metaphase in the root tip cell of a *G. hybrida* regenerant ($2n = 2x = 50$). Bar 20 μm . **d** In vitro microcutting of *Gerbera* cv. Ri4 inoculated with *Agrobacterium rhizogenes* strain 1855. Normal roots (on the right) and transformed roots (on the left) are initiated, respectively, far from and at the level of the inoculated region. Bar 1 cm. **e** Opine electrophoresis at pH 9.2 of *Gerbera jamesonii* root extracts. *S* Standard containing reference opines; *m* mannopine; *a* agropine; *aa* agropinic acid; *am* mannopinic acid; *I* roots arising from the inoculated sites; *R* roots arising far from the inoculated regions; *T* control roots on noninoculated microcuttings

Table 4. Effect of addition of thidiazuron to 10 μ M BA on the percentage of explants provided with buds and on the mean number of buds per explant () for several *Gerbera* species

TDZ (μ M)	Species			
	<i>G. viridifolia</i>	<i>G. piloselloides</i>	<i>G. adriana</i>	<i>G. nivea</i>
0	65.6 (2.9)	8.3 (1.7)	75.0 (5.4)	54.0 (3.2)
0.05	60.0 (3.1)	7.1 (2.0)	—	—
0.5	50.0 (3.6)	39.7 (3.7)	—	—

Table 5. Summary of acclimatization and conformity studies conducted on several *Gerbera* species

Species	Rooted shoots (%)	Acclimatized plants	Phenotypic conformity	2C DNA content (pg)
<i>G. jamesonii</i> cl.10	100	100	Yes	5.2
cl.11	50	30	Yes	5.2
<i>G. viridifolia</i>	40	100	Yes	7.8
<i>G. piloselloides</i>	100	100	Yes	7.8
<i>G. adriana/nivea</i>	—	—	—	5.2

mother plants and regenerants of the same species, revealing that cell differentiation in *Gerbera* occurred without endopolyploidization.

The absence of chromosomal variants demonstrated that clonal bud regeneration could be achieved through culture of juvenile leaf explants excised from in vitro shoot clusters, although the danger of more cryptic modifications of the genome should not be excluded.

2.3 Cytokinin Metabolism

Comparing the pattern of benzyladenine metabolism in the shoots of a range of species cultured in vitro, it was found that *G. jamesonii* behaved particularly, accumulating much more glucosides than other species (Blakesley and Constantine 1992).

Only traces of free BA were present in the shoots (Blakesley et al. 1991). Although little is yet understood about the regulation of cytokinin conjugation, it could be expected that differences in shoot organogenic responses among the various *Gerbera* species and between developing and fully expanded in vitro leaves could be related with differences in exogenous cytokinin uptake and subsequent metabolism.

We recently quantified endogenous cytokinins and IAA in leaf explants of *Gerbera hybrida* clones on regeneration medium (Reynoird, 1996) and found that cytokinin contents at the time of excision had no effect on reactivity. Free zeatin accumulated in the course of bud induction in young leaves of reactive clone whereas increase of inactive cytokinin O-glucosides

occurred in less reactive explants. These results focused on the importance of active endogenous cytokinin levels on regeneration. On an opposite side, only weak differences occurred in metabolism of exogenous BA among clones.

Implication of IAA was also pointed out since strong accumulation was noted for a recalcitrant clone (Reynoird et al., in preparation).

2.4 Genetic Transformation

Following inoculation with wild *Agrobacterium rhizogenes* strains 1855, 8196 and 2659 or with a binary vector bearing both pGV 3850 and pRi 15834 (strain C; construct from A. Petit, ISV – CNRS, F-91198 Gif-sur-Yvette) on wounded petioles of in vitro shoots of *G. jamesonii* cvs. Ri4, Pascal, Rebecca, Joyce, and Terravisa, Caillard (1987) and Caillard et al. (1987) obtained normal and transformed roots (Fig. 2d) containing characteristic opines (Fig. 2e). This demonstrated for the first time that genetic transformation was possible for *Gerbera* using *Agrobacterium*-derived vectors.

The first *Gerbera* plants derived from *Agrobacterium*-mediated gene transfer were obtained recently (Elomaa et al. 1993). Petioles of the red variety *Terra regina* were cocultivated with a disarmed *Agrobacterium tumefaciens* vector containing a nearly full-length antisense cDNA encoding *Gerbera* chalcone synthase (*g chs 1*) under the control of the heterologous CaMV 35S promoter and a *nos-nptII* marker. The transformed cells were regenerated into flowering plants. Four lines of transformants were shown to have integrated both *nptII* and *gchs1* genes with various copy numbers. Transfer of the antisense *g chs 1* into the red-colored *Terra regina* appeared to inhibit anthocyanin accumulation in the petals of two transformants, resulting in pale pink and cream phenotypes.

Although not published, gene transfer was also demonstrated on *G. jamesonii* cv. Frithy, using a combination of adventitious shoot regeneration from fragmented shoot apices cocultivated with pBI 121 harbouring the *gus* reporter gene in LBA 4404 (Kaul, unpubl. reported in Hutchinson et al. 1992).

Recently we obtained shooty tumors from expanded leaves for two clones of *G. hybrida* using vectors derived from *A. tumefaciens* 82139 – a strain that was demonstrated as able to induce shooty tumors on several hosts (Miranda Brasileiro et al. 1991) – and harboring the *gus* reporter gene inside the wild T region (Azmi et al. 1996). Bud regeneration which occurred from tumors was correlated with a dramatic increase of active zeatin riboside content (Reynoird, 1996). GUS localization indicated also that these tumors were chimeric with normal and transformed sectors and that the buds regenerated were normal. Recently we regenerated buds harbouring the *rolC* gene from developing leaf explants.

2.5 Miscellaneous

In vitro-micropaginated shoots of *G. jamesonii* have been irradiated by X-ray (Walther and Sauer 1991) or γ^{60} Co-ray (Dubuc et al. 1987). The mutative changes could be classified according to decreasing frequency: length of stalk, length or width of ligules, color of ligules, size and shape of leaves. Chemical mutagens such as ethylmethane sulfonate could also increase the variability (Schiva et al. 1984). To date, the obtained mutants have been neither propagated nor commercialized.

The allogamous species related to *G. jamesonii*, such as *G. aurantiaca* or *G. viridifolia*, intercrossed easily (Rongeot et al. 1989). Hybridization between distant or autogamous species (*G. nivea*, *G. kunzeana*) appeared more difficult and often needed in vitro embryo rescue. In this way, *G. kunzeana* \times *G. jamesonii* hybrids were obtained by Rongeot (1989). Reciprocal crosses have never been successful.

3 Summary and Conclusions

Mass micropropagation of *Gerbera* has been applied on a commercial scale for almost 15 years. Fifteen to 20 million plants are produced annually, mainly to satisfy the European demand. An increasing part of this production is being transferred to countries with low labor costs. Direct ex vitro rooting could represent the main technical change and important cost savings, but is not yet available for all the clones. To date, no major problem of nonconformity has been pointed out. Adventitious budding might therefore represent an efficient alternative, provided that the phenotype and flower production of the regenerants be maintained. Bud regeneration has been achieved through culture of apices and petioles excised from in vitro plantlets, avoiding the stress of decontamination. The capacity of regeneration from leaves was correlated to their stage of differentiation and strong genotype effects were pointed out. The differential behavior between easy and recalcitrant genotypes could be related to the differences in cytokinin uptake and metabolism. Determination of nuclear DNA contents demonstrated the nonpolysomatic nature of gerberas and the absence of gross chromosomal abnormalities in the regenerants. The capacity of immature leaves and petioles for regeneration has also begun to be exploited for creation of transgenic regenerants by cocultivation with *Agrobacterium*-derived vectors. On the basis of efficient regeneration systems that yield a consistent number of regenerated buds, it could be expected that gene transfer using genes of economic value is going to be extended to *Gerbera* in the near future. Genes involved in new colors, delay in flower senescence, floribundity, accelerated flowering, dwarfism, and resistance to pathogens are among the best candidates for such challenges.

4 Protocols for Micropropagation

4.1 Micropropagation via Axillary Branching

1. Establishment. *From shoot tips*: grow the plant in a pot, uncovering the upper part of the rhizome and avoiding excess watering for few days before establishment. Divide and strip the plants, keeping only the last unexpanded leaves and the shoot tips, gently brushing after each removal. Reduce the subjacent rhizome to a 2-cm piece and peel off the periderm. Under the laminar, gently remove the last unexpanded leaves, flaming briefly several times to eliminate hairs.

With the scalpel, transfer the 2-mm-long shoot tip explants on the multiplication medium at 25°C supplemented with 2.5 μ M kinetin, 2.5 μ M BA, 2.5 μ M IAA under a 16-h photoperiod (60 μ mol/m²/s from Sylvania Grolux 115W tubes) for 4–6 weeks. Multiplication medium: 145 mg/l KNO₃, 1225 mg/l NH₄ NO₃, 375 mg/l Ca(NO₃)₂, H₂O, 450 mg/l Mg SO₄, 7 H₂O, 225 mg/l CaCl₂ H₂O, 225 mg/l KH₂PO₄, 40 mg/l NaH₂PO₄, 25 mg/l KCl, MS micronutrients and vitamins, 100 mg/l glutamine, 20 mg/l adenine sulfate, 30 mg/l sucrose, 8 g/l Difco-Bacto agar.

From immature capitula: surface sterilize the immature inflorescences (0.5–0.7 cm in diameter) for 30 s in 70% ethanol then 90 min in 1.5% sodium hypochlorite and wash 45 min in sterilized tap water; remove the involucral bracts, divide each capitulum into five to ten explants and inoculate on multiplication medium, supplemented with 2.5 μ M kinetin, 2.5 to 5 μ M BA, and 2.5 μ M IAA for 8 to 12 weeks under the conditions above.

2. Rooting. After division, each shoot provided with two leaves is transferred for 3 weeks under the same conditions on half-strength MS medium supplemented with 0.25 to 1 μ M NAA or 2.5 to 7.5 μ M IBA or IAA, depending on the clone. IBA and NAA lead to numerous short and thick roots, while IAA leads to few long and thin roots.

4.2 Micropropagation via Bud Regeneration

After subculture on multiplication medium, excise the leaves, taking care to exclude axillary buds at their proximal end. Place the abaxial side of leaves in contact with the multiplication medium supplemented with 10–25 μ M BA and 0.5–2.5 μ M NAA in Petri dishes. For the fully expanded leaves supplement also the multiplication medium with 0.5 μ M TDZ. Place the Petri dishes under the conditions described above for 4–6 weeks, then transfer the regenerating calli on the multiplication medium containing low hormone levels, i.e., 2.5 μ M kinetin, 2.5 μ M BA, and 2.5 μ M IAA.

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I.12 Micropropagation of *Helianthemum almeriense*

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1 Introduction

1.1 Botanical Characteristics and Distribution

Helianthemum almeriense Pau in Mem. Mus. Ci. Nat. Barcelona, Ser. Bot. 1 (3): 11 (1925) = *H. leptophyllum* auct., = *H. pilosum* auct., non (L.) Mill. Its common names are: Spanish: Mata turmera, Perdiguera, Tamarilla blanca (Turma = common name of *Terfezia claveryi*).

The genus *Helianthemum* Mill (Cistaceae family) includes shrubs or herbaceous plants, perennial or annual, and is well represented in the semiarid Mediterranean region. Etymologically, *Helianthemum* means: hélios (gr. m) = sun; and ánthemon (gr. n) = flower; that is to say *Helianthemum* directs its flowers towards the sun (López-González 1993).

H. almeriense Pau is a species whose distribution is restricted to the southeast of Spain and the north of Africa (Morocco), and which establishes a mycorrhizal symbiosis with *Terfezia claveryi* Chatin, commonly known in the area as turma, desert truffle, or criadilla (Fig. 1C,D). This fungus is a great local delicacy and is appreciated throughout the Arabian countries.

It is a shrub, 10–15 cm tall, fruticose intricated raceme and with more or less divaricated branches (Fig. 1A). The shoots are divaricated or erect, glabrous to tomentous and reddish brown in color. The leaves are elliptical to oblong-lanceolated or linear-lanceolated, obtuse, flat, or more rarely with a revoluted margin, from glabrous to pubescent star-shaped, possibly becoming tomentous on the reverse side; the leaf (blade) is from 2–15 × 0.6–3.5 mm (Fig. 1B). Inflorescences are simple, lax, with two to ten flowers. The floral buds are ovoid or conical, pointed, with a twist at the apex. The internal sepals, 4–8 mm long, are ovate-elliptical, membranous, glabrous, or even pubescent star-shaped, generally of a purple color, with prominent ribs, rarely setose. The external sepals are linear-spatulate to elliptical, three times smaller in size than the internal ones, glabrous, and green in color. The petals, 8–12 mm long, are obovate-flabelliform, sometimes retuse, white, and more rarely pink, maculate (Fig. 1A,B). The fruit is a capsule from 4.5–7 mm long, similar to or smaller than the calyx, globose or elliptical, densely pilose, polysperm. The

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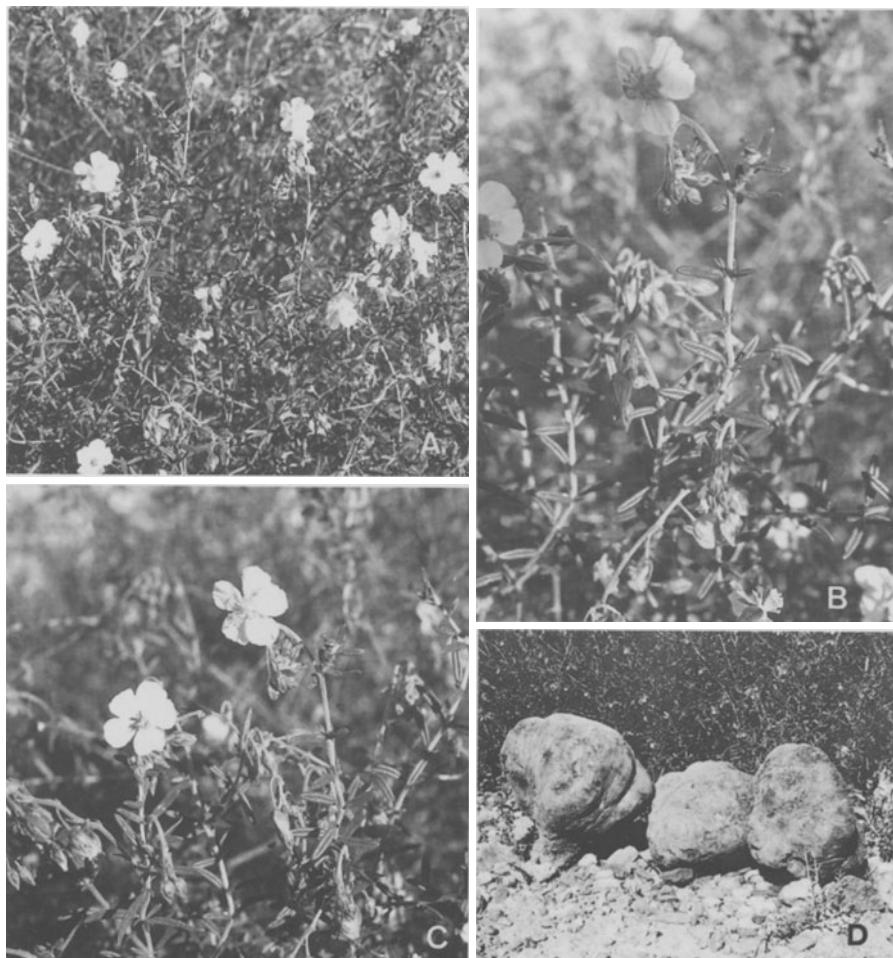


Fig. 1A–D. *Helianthemum almeriense*. **A** A 2-year old shrub. **B, C** Details of flowers. **D** Sporocarps of *Terfezia claveryi*

seeds, 1.2–1.5 mm long, are chestnut brown, or dark reddish brown. $2n = 20$, $n = 10$.

According to López-González (1993), the plant appears in open places, in dry, stony, limestone, mica, marl, or marl with gypsum soils, even in sandy terrain, between 0 and 500 m altitude. It is well represented in the arid southeast of the Iberian peninsula, particularly in the provinces of Alicante, Almeria, Granada, Málaga, and Murcia. It has also been found in Morocco.

1.2 Uses and Propagation

No specific use is known, except that it is a mycorrhizal plant with *Terfezia claveryi* and other edible fungi [*Melanogaster variegatus* (Vitt.) Tul. & Tul., *Picoa juniperi* Vitt., etc.].

The germination of the seed is usually erratic. Micropagation has been successfully applied for a large number of plants of agricultural and sylvicultural interest (see Bajaj 1992). On the other hand, it has been demonstrated that controlled mycorrhization of plants of sylvicultural interest has helped them to adapt themselves better to edaphic and climatic conditions during reafforestation and to improve their growth.

Studies on micropagation protocols are scarce when we talk about mycorrhization. Coupling both techniques would allow us to select the fungus strains and type of explants which would be ideal in different culture conditions for different field conditions.

Micropagation and mycorrhization studies on *Helianthemum almeriense* are presented because of the double interest of this species. On the one hand, because we are dealing with a shrub species, which is well adapted to the semiarid conditions of the southeastern Iberian peninsula, and because some species develop in saline soils, with high concentrations of gypsum, making it an ideal candidate for reafforestation uses in these areas; and, on the other hand, because it belongs to the Cistaceae family and establishes mycorrhizal symbiosis with species of the *Terfezia* genus which, together with *Balsamia*, are the hypogeous fungi called desert truffles or turmas.

Terfezia claveryi was selected as the symbiont fungus, as it is the most frequent in marl with gypsum soils of the semiarid western Mediterranean area and is symbiotic to several species of the *Helianthemum* genus, both annual and perennial (Honrubia et al. 1992). Therefore, the fungal ecotypes, and those of the symbiont plants, respond to well-marked xerophytic conditions.

The desert truffle market extends to all the coastal countries of the Mediterranean and the Persian Gulf. Saudi Arabia and Kuwait are the main consumers, but they are eaten in Turkey, Egypt, Italy, and also Morocco. In Spain it is centered fundamentally at the family level, in the region between Murcia-Almeria-Granada, and in western Andalucia and Extremadura, depending on the fungal species. The culinary and commercial value of the turma does not seem to vary among the species. In the southern peninsular and Barcelona markets it can be found at prices higher than those reached by other well-known fungi, such as *Lactarius deliciosus* Fr.

For this reason, micropagation is necessary, not only as a variant to the traditional method, but also to be able to control and better understand the nutritional requirements, thus enabling good mycorrhization. This mycorrhization would help plants to adapt better to post vitro conditions during the acclimatization phase and later to field conditions, and, at the same time, provide a commercially interesting end product.

2 Micropagation

2.1 Material and Methods

2.1.1 Plant Material and Culture Media

Mature seeds of *H. almeriense* were collected from a field close to the Campus Universitario in Espinardo (Murcia, Spain). They were surface sterilized by pretreatment for 10 s in a 70% ethanol solution, followed by treatment for 20–30 min in a 10% commercial bleach (Domestos) solution, followed by rinsing in 80% ethanol for 10 s and three 5-min rinses in sterile distilled water, and then transferred to basal media.

Three culture media were screened in the initial investigations, but only one was used in the bulk of the experiments dealing with organogenesis and regeneration, those of Murashige and Skoog MS (1962), Schenk and Hildebrandt SH (1972), and Gresshoff and Doy (1972). The pH was adjusted to 5.8 before autoclaving. The media were gelled with 0.7% Panreac agar and autoclaved for 20 min at 120 °C and 103 kPa.

Screening of media was done using shoot tips (1 cm) and nodal segments (0.5–1 cm) from the in vitro-germinated seedlings. Cultures of the three media included 1.39 μ M of kinetin. Newly formed buds were measured and transferred to a fresh medium after 4 weeks on these media. Explants were subcultured up to six times. Duncan's statistical test (Duncan 1955) was applied to the results. The cultures were incubated in a growth cabinet at 22 \pm 2 °C, at a light intensity of 40 μ mol/m²/s for a 16-h photoperiod.

2.1.2 In Vitro Multiplication and Rooting

For shoot proliferation trials, MS medium was used, being selected as the best in the media test. Shoot tips and nodal segments (of 1.5–2 cm length and two or three nodes each one) were cultured on this medium containing different concentrations of BA, kinetin, and 2iP, with or without 0.27 μ M of NAA (Table 2).

After 1 year in culture, the agar concentration was increased to 0.8% to avoid tissue hyperhydration, which usually appears after numerous subcultures on medium with cytokinins. Thirty cultures per treatment were made and experiments were repeated at least three times. Duncan's statistical test was applied to the obtained data.

Following shoot multiplication and elongation, 15–20-mm shoots were transferred to root induction medium consisting of MS salts at several strengths (1x, 1/2x, 1/4x) with or without activated charcoal (2 g/l), 3% sucrose, and 0.7% Panreac agar. Forty-eight explants were used per treatment and experiments were repeated five times.

After 6 weeks, plantlets with well-developed roots were potted in a peat-sand-vermiculite mixture (3:1:1, v/v). The plantlets were gradually exposed to

reduced relative humidity by progressively removing a plastic cover during a period of 2 to 3 weeks in the greenhouse.

2.1.3 Plantlet Mycorrhizal Inoculation

Rooted plantlets were inoculated in vitro with mycelium of *Terfezia claveryi* on MH medium, following the procedure described by Morte and Honrubia (1994). Mycelium of *T. claveryi* was obtained from fruitbody tissues and cultured on MMN agar medium (Marx 1969) at pH 8.

After in vitro mycorrhizal colonization, plantlets were transferred to pots containing a mixture of peat-vermiculite-sand (1:1:1, v/v). This substrate was sterilized by autoclaving for 60 min at 100 °C, three times on alternative days. These plantlets were acclimatized under greenhouse conditions by reducing relative humidity for a period of 2 to 3 weeks.

Fungal colonization was assessed on cleared and stained root samples (Phillips and Hayman 1970). The percentage of fungal root colonization was estimated according to the gridline intersect method (Giovannetti and Mosse 1980) under a stereomicroscope.

2.2 Results and Discussion

2.2.1 Media

After six subcultures of 4 weeks each, in the three different tested culture media containing 1.39 μ M kinetin. MS mineral solution appeared to be more suitable for this species when compared with SH and GD media, as the number of buds per explant was significantly improved (Table 1). Moreover, explants on MS medium grew better than on SH and GD media, where explants showed apical shoot necrosis and yellow leaves, with death of most explants. GD medium was the worst. This suggests that *H. almeriense* requires a rich nutrient medium for in vitro development.

2.2.2 In Vitro Multiplication

The propagation system followed with this species was principally the formation of axillary buds and the culture of nodal segments. The incorporation of cytokinins in the medium caused, to a great extent, the elongation of the explants, which allowed them to be cut into several nodal segments (Fig. 2A). The cultures established from both apical and nodal segments had the same proliferation rate in the first subcultures. For this reason, the following data are unified and apply to both types of explant.

The production of axillary buds and the formation of new nodal segments started at 2 weeks postculture of the explants in MS medium with cytokinins. The maximum number of shoots per explant was obtained with 0.46 and

Table 1. Effect of different media (with $1.39\mu\text{M}$ kinetin) on multiplication rate of shoot tips and nodal segments of *H. almeriense*

Culture media	No. of shoots per explant
GD	0.35 a
SH	1.37 b
MS	3.94 c

Values followed by the same letter in a column are not significantly different ($P \leq 0.05$), as determined by Duncan's test.

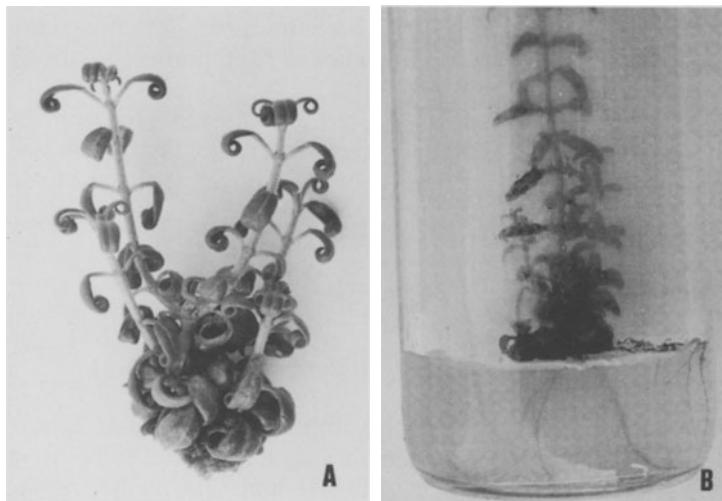


Fig. 2A-B. Stages of micropropagation in *H. almeriense*. **A** Proliferated shoots of a 3-month-old explant on MS basal medium with $0.46\mu\text{M}$ kinetin. **B** Root formation at the base of a 1-year-old plantlet. (A,B Morte and Honrubia 1992)

$0.93\mu\text{M}$ of kinetin (Table 2). These concentrations were fairly low, but allowed good growth of the shoots. In the case of *H. almeriense*, kinetin was more effective than BA or 2iP in the production of shoots. Elevated concentrations of BA only ($1.78, 2.66, 3.55\mu\text{M}$), or in combination with $0.27\mu\text{M}$ of NAA ($0.44, 0.88, 1.33\mu\text{M}$) produced a decrease in the number of shoots. When $3.55\mu\text{M}$ of BA and $1.33\mu\text{M}$ of BA were used with $0.27\mu\text{M}$ of NAA, a callus was formed in the base. The phytohormone 2iP produced chlorosis and necrosis in the majority of the cultures, with the lowest rate of multiplication (Table 2). This hormone had the same effect in *Cistus x purpureus* (M'Kada et al. 1991).

The multiplication rate decreased considerably after the fourth subculture and reached a minimum in the sixth (0.91 shoots/explant; Fig. 3).

Parallel to this decrease, an increase in hyperhydricity was observed, reaching 65%. Debergh et al. (1992) explained that the susceptibility of the

Table 2. Influence of BA, kinetin, 2iP, and NAA on number of microshoots produced from shoot tips and nodal segments of *H. almeriense* cultured on MS. (Morte and Honrubia 1992)

BA	Kinetin	2iP $\mu\text{M/l}$	NAA	No. of shoots per explant
—	—	—	—	0 a
0.22	—	—	—	2.48 abc
0.44	—	—	—	2.23 abc
0.88	—	—	—	2.29 abc
1.33	—	—	—	2.79 abc
1.78	—	—	—	0.99 ab
2.66	—	—	—	1.31 ab
3.55	—	—	—	1.66 ab
0.22	—	0.27	—	2.91 bc
0.44	—	0.27	—	2.33 abc
0.88	—	0.27	—	2.32 abc
1.33	—	0.27	—	1.86 abc
—	0.46	—	—	7.72 d
—	0.93	—	—	6.12 d
—	1.39	—	—	4.08 c
—	—	0.49	—	1.33 ab
—	—	0.98	—	0.72 a
—	—	1.39	—	0.76 a

Data in a column followed by the same letter are not significantly different ($P \leq 0.05$) as determined by Duncan's test.

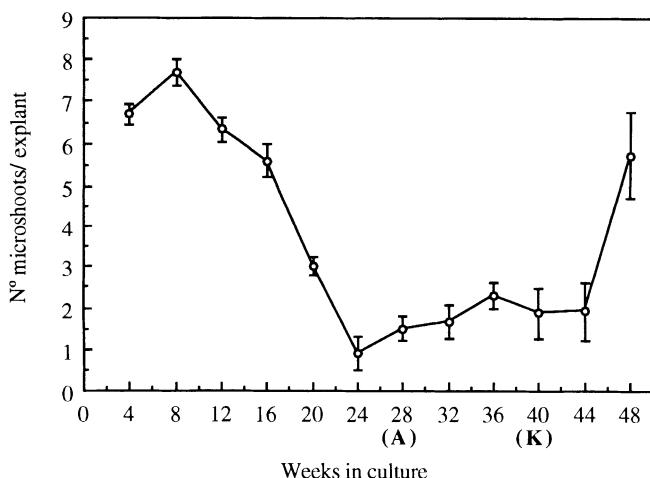


Fig. 3. Variation of multiplication rate of *H. almeriense* explants cultured on MS medium with $0.44 \mu\text{M}$ kinetin, for 48 weeks. A Addition of 0.8% agar; K addition of $18.58 \mu\text{M}$ kinetin

explants to hyperhydrate varied, among other factors, with the number of subcultures. When hyperhydricity of the tissues appears in the propagation scheme, multiplication rate, rooting, and survival decrease in the affected shoots when they are transferred to the greenhouse (Von Arnold and Eriksson

1984). Kevers et al. (1984) and Debergh et al. (1992) summarized the problems of hyperhydricity of in vitro-cultured plants, and reached the conclusion that there are multiple factors implicated in this phenomenon. Among them can be cited the hydric potential of the medium, correlated to the concentration and type of agar; at low concentrations hyperhydricity is induced, and different kinds of agar give different results. It is, therefore, necessary to compensate for this to obtain the required firmness of the gel (Debergh 1983; Ziv et al. 1983, Pasqualetto et al. 1988).

It has been demonstrated that both the multiplication rate and the aspect of the shoots can be manipulated by increasing the concentrations of agar in the culture medium (Romberger and Tabor 1971; Ziv et al. 1983; Von Arnold and Eriksson 1984; Debergh et al. 1992).

For the above-mentioned reason, it was decided to increase the concentration of agar in the medium from 7 to 8 g/l. This was done in the seventh subculture. The multiplication rate improved considerably from the sixth (0.91) to the ninth (2.3) with this process. Nevertheless, the multiplication levels continued to be lower with respect to the initial ones, and, furthermore, in the tenth subculture a slight decrease, from 2.3 to 1.88, was observed. This decrease could have been due to a fall in the susceptibility of the explant to the kinetin concentration in the medium, apart from the fact that if the concentration of agar is increased, it becomes more difficult for the explant to establish contact with the medium, which limits the absorption of the compounds (Pierik 1987). Among these, the growth regulator and, in the case of *H. almeriense*, kinetin.

This "habituation" of the explants to the growth regulators, after several subcultures, consists of a considerable increase in the need for regulators as the culture becomes older. This habituation does not generally constitute a permanent change, since if the plants formed from habituated tissues and explants are then isolated, these require regulators again. In other words, habituation changes are epigenetic in nature.

To reactivate the *H. almeriense* explants used to a concentration of 0.46 μ M of kinetin, the concentration was raised in the medium, incorporating 18.58 μ M of kinetin in the tenth subculture and returning to the initial concentration of 0.46 μ M in the following ones. Reactivation did not occur until the 12th subculture; the rate of multiplication went from 1.88 to 1.92 in the 10th and 11th, respectively, then increased to 5.73 in the 12th (Fig. 3).

For shoot elongation, it was not necessary to change the culture medium, as the shoots elongated in the same multiplication medium. This was due to the propagation system used: the formation of nodal segments, which gave as a result explants up to 10 or 11 cm long in the multiplication stage.

On the other hand, the type of cytokinin used, and the relatively low concentration, allowed the axillary ramification-formed shoots to reach, at the end of the multiplication stage, an adequate length (2.5–3 cm) for their direct use in the following rooting stage. Therefore, in *H. almeriense*, the shoot multiplication rate is directly related to shoot elongation and number of nodal explants available in each species.

2.2.3 Root Induction and Development

Spontaneous rooting was observed during the multiplication stage in cultures with a low level of cytokinins, which was 81% in medium with 0.46 μM of kinetin, 65% with 0.44 μM of BA, and 24% with 0.49 μM of 2iP (Fig. 2B). This spontaneous rooting was mainly seen up to the sixth subculture. After this, it was lower (approximately 40% in medium with 0.46 μM kinetin), although it was still seen in explants cultured in this multiplication medium after 2 years of culture.

The problem in rooting was that in the majority of cases it was accompanied by the formation or abnormal growth of the epidermal cell layer of the roots. This meant they did not take when placed in pots. To avoid this, and to improve the rooting percentage, the macronutrient concentration was diluted in the medium, and active charcoal was incorporated. Owing to the ease with which *H. almeriense* explants root, it was not necessary to use auxins in the medium at this stage. In our case, the rooting percentage improved to 92% on diluting one quarter or one half of the macronutrient concentration (Fig. 4). M'Kada et al. (1991) showed that the dilution of the macronutrient concentration improved rooting of explants of *Cistus x purpureus*.

On the other hand, the addition of active charcoal reduced rooting, which is the opposite of observations by Cheema and Sharma (1983) and M'Kada et al. (1991). The negative effects of active charcoal on rooting with *H. almeriense* could be related to the adsorption of endogenous auxins which promote

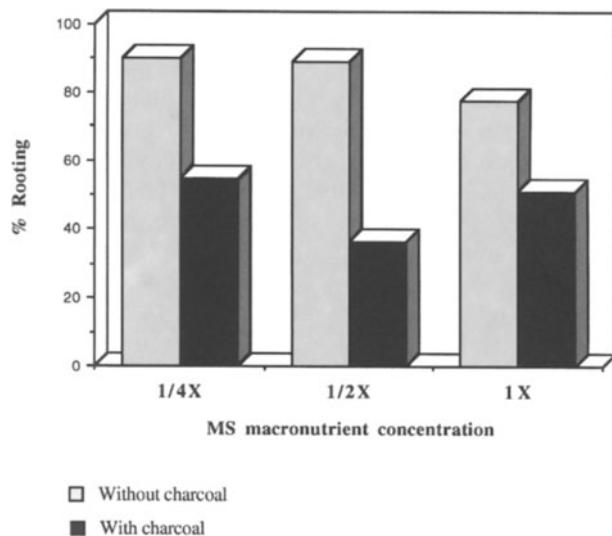


Fig. 4. Influence of concentration of macronutrients and addition of charcoal on in vitro rooting of *H. almeriense* explants, on medium without growth regulators after 4 weeks. (Morte and Honrubia 1994)

rooting, since one of the effects of active charcoal is that of adsorbing organic compounds.

2.2.4 Transfer to Soil

Ten days after transferring the plantlets to a substrate mixture of peat, sand, and vermiculite, the humidity was slowly reduced by opening small holes in the plastic bags which covered the containers. At 20–22 days the plastic covering was completely removed. At the end of 1 month, approximately 95% of the plantlets survived.

Between the 6th and 8th week, the acclimatized plantlets were transferred to field conditions in a control plot situated near the greenhouse, a natural habitat of these species. Here, a high survival rate was observed, varying between 75 and 85%, and they showed normal development and flowering during the 2 years in which they were observed.

Flowering was observed in some plantlets even under greenhouse conditions, before their transference to the field. Normal flowering could be favored by the low hormone concentration level used at the propagation stage.

2.2.5 Mycorrhization

The mycelium grew from the surface of the agar pieces, which served as an inoculum, towards the interior of the culture medium in the test tube, within 2 weeks. This mycelial growth into the agar of the tube allowed the mycelium to perfectly colonize the entire root system of the plantlet (Fig. 5A). The mycorrhization percentage obtained varied from 61–75% after 2 months in culture. This percentage was very similar to that obtained in MMN culture (Morte et al. 1994). However, in contrast to MMN mycorrhization, in the MH medium, there were no problems with in vitro survival during the time that the rooted plantlets took to mycorrhize. This could be due to the fact that its composition was more similar to that of MS micropropagation medium and had a greater number of nutrients than MMN medium (Morte and Honrubia 1994).

Although there were no survival differences between the mycorrhizal and control plantlets, growth differences occurred. *T. claveryi* produced a growth stimulation in the plantlets and, in comparison to the nonmycorrhizal controls, the increase in height of the mycorrhizal plants was practically double (Fig. 6). This stimulation could be attributed to the widely studied effect that the formation of mycorrhizas has on plants. Moreover, the slightly lower pH in the MH medium (pH 7), in comparison with the MMN medium (pH 8) allowed a better plant development in vitro, since pHs above 7 generally inhibit plant growth in vitro (Pierik 1987).

With respect to the morphology of the mycorrhiza formed, it was characterized by a discontinuous mantle of lax hyphae around the mycorrhizal root (Fig. 5B), the opposite to that seen when the synthesis was performed in MMN

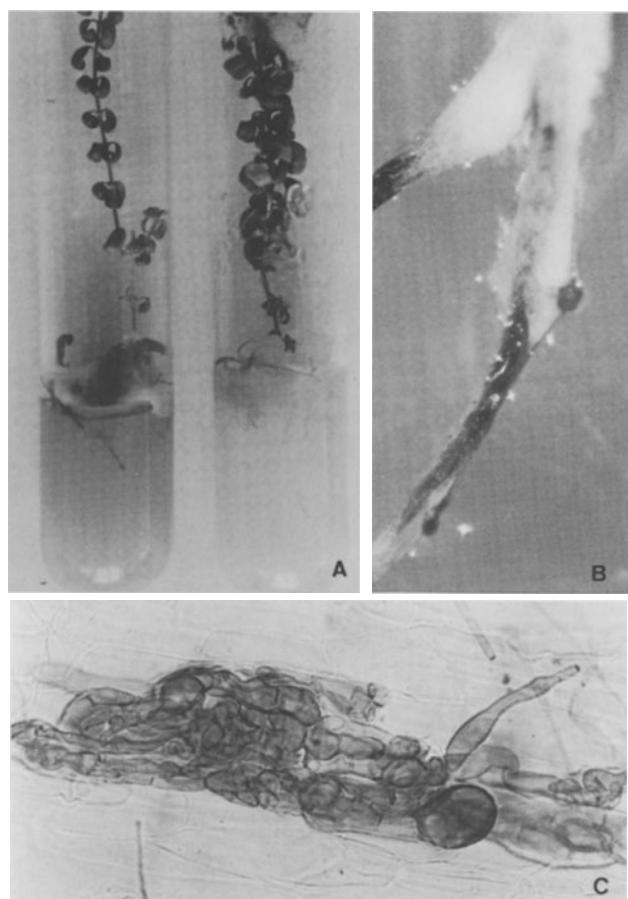


Fig. 5A–C. Mycorrhization of micropropagated *H. almeriense* plantlets with *T. claveryi*. **A** In vitro system for inoculating micropropagated plantlets with *T. claveryi* mycelium on MH medium. **B** A discontinuous mantle of lax hyphae around the mycorrhizal root. **C** Longitudinal section of infected root; the hyphae form coils that fill the host cells, showing a bead shape. 40x. (C Morte and Honrubia 1992)

medium (Morte et al. 1994) and in substrate with sporal suspension (Cano et al. 1991). The presence of this mantle of hyphae was probably due to the better growth of the mycelium of *T. claveryi* in the MH medium and the small space in the test tube, which helped to concentrate the hyphae around the roots. The hyphae formed coils which occupied the whole cell lumen (Fig. 5C). The rest of the structures formed were similar to those of an ectendomycorrhiza like those described with the MMN medium by Morte et al. (1994) and by Fortas and Chevalier (1992).

In vivo mycorrhization has the disadvantage that the actual soil conditions cannot be totally controlled (pH, quantity of available nutrients, conductivity, etc.), which can limit the growth of some of the symbionts or the end product

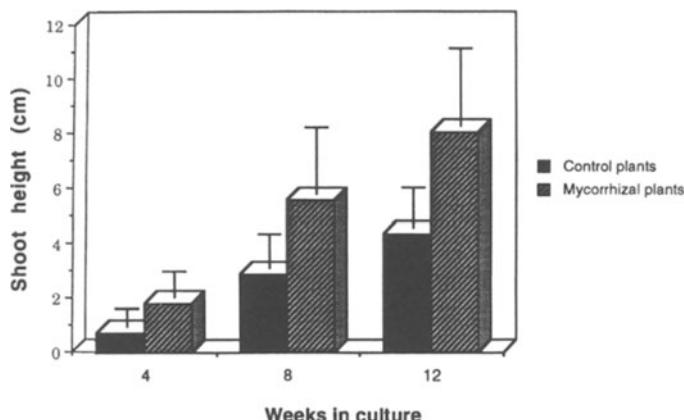


Fig. 6. Effect of in vitro inoculation with *T. claveryi* on shoot height of micropropagated *H. almeriense* plantlets on MH medium. Bars SE of means. (Morte and Honrubia, Patent P9402430, Madrid)

of their mycorrhization. However, in vitro mycorrhization allows us to control the optimum conditions of symbiosis and growth of the fungal and plant species, as well as to select the fungus strain and explants most appropriate to culture conditions under specific field conditions. The MH culture medium (Morte and Honrubia 1994) solved these problems of nutritional requirements so that both symbionts grew correctly, and, at the same time, a nutritional equilibrium was created which allowed its symbiotic association.

Although there are several reports on in vitro mycorrhization, few have used agar as substrate (Malajczuk and Hartney 1986; Tonkin et al. 1989; Roth-Bejerano et al. 1990). Some authors (Rancillac 1982; Douglas et al. 1989) consider that agar as a substrate does not help with mycorrhizal synthesis, because it produces a lack of gas exchange which is necessary for thus symbiosis. However, the medium used in this experiment was designed with agar because, apart from having obtained a high mycorrhization percentage, it enabled us to observe the growth of the mycelium on the root and, thus, the start of mycorrhization. The agar also turned out to be more easily manageable than any other type of substrate.

The plantlets that survived the in vitro mycorrhization were transferred to pots. They acclimatized with survival percentages similar between mycorrhizal plants and controls.

2.3 Commercial Aspects

Some commercial aspects can be derived from the use of this species as a mycorrhizal symbiont of *Terfezia claveryi*. As a result, we have designed a patent (Morte and Honrubia 1994) describing a method for the in vitro myc-

orrhizal symbiosis between these species in an agar MH medium (Morte and Honrubia medium) specially made for the mycorrhization. The in vitro system enabled us to obtain effective mycorrhization that is rapid (with mycorrhization percentages of 75% in 2 months), reliable (MH medium allowed a good plant and fungus growth which facilitated their following growth and survival in pots), controlled (the agar permits the observation of the mycelium growth around the root, the detection of the beginning and development of the mycorrhization and it is more easily manageable than other kinds of substrates), and at a low cost (the test-tube system permits the mycorrhization of a large number of plants in a very reduced space and with small substrate and inoculum amounts per plant).

3 Summary and Conclusions

A protocol of in vitro culture has been described for *H. almeriense*. Shoot tips and nodal segments were excised from seedlings grown in vitro. Outgrowth of axillary buds was achieved on Murashige and Skoog (MS) medium supplemented with $0.46\mu\text{M}$ kinetin without auxins. The multiplication rate decreased considerably after the fourth subculture. Parallel to this decrease, an increase in hyperhydricity was observed. This problem was eliminated by increasing agar concentration in the medium from 7 to 8 g/l. To avoid habituation of explants to the growth regulator, kinetin concentration in the medium was raised to $18.58\mu\text{M}$ in the tenth subculture and returned to the initial concentration in the following ones.

Spontaneous rooting was observed at a low cytokinin concentration until the sixth subculture. The rooting percentage increased to 92% via macronutrient dilution. The rooted shoots were grown in a peat-sand-vermiculite mixture.

Micropropagated plantlets were mycorrhized in vitro with *Terfezia claveryi* on MH agar medium at pH 7.0. The mycorrhization rate was about 75% after 8 weeks. This mycorrhization produced a growth stimulation in the plantlets. *T. claveryi* formed ectendomycorrhizas with a discontinuous mantle of lax hyphae.

4 Protocol

1. Initial explants: shoot tips and nodal segments of in vitro germinated seeds.
2. Culture conditions: $22 \pm 2^\circ\text{C}$, $40\mu\text{mol/m}^2/\text{s}$ Growlux fluorescent light and 16-h photoperiod.
3. Culture medium: Murashige and Skoog (MS) (1962), 0.8% Panreac-agar, 3% sucrose, pH 5.8.
4. Multiplication stage: MS medium with $0.46\mu\text{M}$ kinetin, for 4 weeks, pulse of $18.58\mu\text{M}$ kinetin for 4 weeks when multiplication rate decreases.
5. Rooting stage: 1/4 macronutrient dilution and without plant growth regulators, for 3–4 weeks.
6. Weaning stage: peat-sand-vermiculite mixture (3:1:1, v/v).

7. In vitro mycorrhization: – Inoculum: pieces of mycelium of *Terfezia claveryi* – Substrate: Morte and Honrubia (MH; 1994) agar medium with pH 7.0
8. Weaning after in vitro mycorrhization: peat-sand-vermiculite sterile mixture (1:1:1, v/v).

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I.13 Micropagation of *Leucojum aestivum* L. (Summer Snowflake)

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1 Introduction

1.1 Botany, Distribution, and Importance

Leucojum aestivum L. (Amaryllidaceae) is a perennial bulb plant with linear leaves and is up to 65 cm high (Fig. 1). It exhibits a very well expressed period of physiological dormancy, which starts at the beginning of the summer after ripening of the seeds. The active vegetation period is from February to June.

Propagation by bulblets and seeds takes place in the season of abundant soil water reserves and swappiness of the natural fields (Komendar and Sabadosh 1986). As a result of vegetative propagation, groups of 10–20 bulbs are formed from one initial bulb for a period to 12–15 years (Astadzov et al. 1980).

Leucojum aestivum L. ($2n = 22$, Stephanov 1990) is distributed over boggy and seasonally flooding meadows, at the periphery of swamps, up to 350 m above sea level. It can be found in Atlantic regions and southern Europe, the Balkan Peninsula, the Crimea, the Caucasus, and Central Asia. It has been artificially introduced to central Europe and America. Some fields in Bulgaria are natural reserves.

The plant has a pharmaceutical value, and is rich in alkaloids. Galanthamine, lycorine, lycorenine (Biot 1957; Bubeva-Ivanova and Ivanov 1959; Stephanov 1990), nivalidine (Bubeva-Ivanova 1958), and hypeastrine (Bubeva-Ivanova and Ivanov 1965) have been isolated. Galanthamine is the only substance of the group of Amaryllidaceae alkaloids which has found broad therapeutical application. Its anticholinesterase activity is used in the treatment of nervous disorders (Temkov et al. 1960; Losev and Kamenetskig 1987) Galanthamine also has an antimyasthenic effect and is an antagonist to curare-like drugs (Tanahashi et al. 1990). Its analeptic effect can be clinically used for anesthetic purposes (Cozanitis and Toivakka 1971). For medical treatment, the hydrobromide of the substance is available as Nivalin (Pharmachim, Sofia, Bulgaria) and Galanthamine (Medexport, Moscow, Rus-

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Fig. 1. *Leucojum aestivum* L. plant

sia). The medicines are used for treatment of poliomyelitis and diseases of the central and peripheral nervous system (Davis 1987).

The highest galanthamine concentrations were found in plants of the Amaryllidaceae family, while the members of other families (Agavaceae, Haemodoraceae, and Hypoxidaceae) contained less than 0.01% galanthamine (Poulev et al. 1993; Tanahashi et al. 1990). Poulev et al. (1993) reported an average galanthamine concentration of 1.64% in all 1000 plant samples tested in southern Bulgaria, and even as much as 5% of this alkaloid in some plants. At present, *L. aestivum* is the major source of galanthamine.

1.2 Conventional Propagation and Need for Micropagation

The galanthamine supply for medicine production depends entirely on the natural resources of *L. aestivum* and on the plants growing under field conditions with artificial irrigation.

Seed propagation is another conventional method for commercial purposes. The abundance of soil humidity and 15–20°C optimal temperature are essential for seed germination (Astadzhov et al. 1980).

Leucojum aestivum L. is included in *The Red Book of the Republic of Bulgaria* (1984) as a Euro-Mediterranean species in danger of extinction. The natural growth areas of this medicinally important plant are threatened with extinction (Stoyanov 1973; Stoyanov and Savchev 1964; Dimitrov 1988).

The condition of the natural *Leucojum* fields is deteriorating every year due to large-scale drainage to convert them into agricultural areas, to bulb picking for Nivalin production, and to contamination of the rivers by industrial waste.

The provision of *L. aestivum* L. plants for galanthamine extraction for Nivalin production encounters difficulties due to the resource deficiency. To preserve the natural supply of this plant, cv. Snezhinka was developed and introduced into production (Astadzhov et al. 1980). However, *Leucojum* plant production is very inefficient, due to its need for soils rich in humus and mineral compounds, repeated irrigation, and mechanical difficulties in picking the leaves and stems.

The chemical synthesis of galanthamine-2-O-hemisuccinate has until now been too low-yielding, and the production too expensive to compete commercially with the natural product (Tanahashi et al. 1990). Therefore rapid in vitro micropagation is an alternative to overcome the plant shortage for drug production and also to conserve the endangered species.

2 In Vitro Studies and Micropagation

Hussey (1975) noted that the Amaryllidaceae family is recalcitrant to in vitro regeneration. Atanassov and Kikindonov (1972) established the organogenic ability and vegetative primordium formation of the callus. Chavdarov et al. (1984) investigated the morphogenetic ability of the bulb scales. Popov and Chercasov (1984, 1986) reported organ induction in bulb and chlorophylless leaf explants.

Natural endogenous bulb infection is a great obstacle for the successful development of in vitro micropagation of *L. aestivum*. No promising sterilization of *Leucojum* organs has been achieved. Chavdarov et al. (1984) established that geographic plant origin and the time interval between bulb collection and sterilization significantly influence the extent of disease infection after sterilization.

Popov and Chercasov (1984, 1986) reported that MS medium (Murashige and Skoog 1962) is favorable for in vitro cultivation. Organ induction was achieved on MS agarose medium containing NAA (1-naphthaleneacetic acid), BAP (6-benzyl-aminopurine), and kinetin at a concentration of 0.5 or 1 mg/l. The higher NAA and BAP concentrations (3–5 mg/l) suppressed organogenesis and induced callus formation in many cases. The reduction of phytohormone concentration to 0.1 mg/l stimulated plant development.

The optimal cultivation temperature for most species is about 25°C. However, Yeoman (1973) reported that the most favorable culture temperature in some bulb species is considerably lower, 15–18°C. Popov and Chercasov (1986) established root formation in explants of *Leucojum aestivum* only at 5°C.

2.1 Sterilization of Material

Attempts to develop optimal sterilization conditions for leaves and bulbs of *L. aestivum* showed uneven and very high endogenous infection of the initial plant material. The percentage of infected bulb explants was much higher compared to those excised from above ground organs. Explants from bulblets and the central part of the main bulb were least infected. The infection of the bulb scale a spread from the bulb core to the outer cover, and the upper parts of leaf explants were more infected than young leaves.

A three-factor experiment using Sadovski's composition scheme (1986) with dispersion and regression analysis of the results obtained was carried out to optimize the sterilization conditions.

Factor A: length of period between collection of plant material and in vitro inoculation.

Factor B: duration of washing with running water before sterilization.

Factor C: exposure to 0.1% HgCl_2 . We chose the following grades for the three factors:

Grades	Factor A (days)	Factor B (h)	Factor C (min)
0	0	0	0.5
1	7	4	1
2	14	16	2
3	28	24	4
4	42	48	6

Sadovski's composition scheme, including 11 variants (Table 2), allows the determination of the optimum grade for each of the three factors and avoids the repetition of all possible 125 variants ($5^3 = 125$). Table 1 shows the effect of each of the three factors tested.

Each of the 11 variants was repeated twice with plant material originating from one and the same population. The control variant 000 was inoculated immediately after the plants were collected, without washing with running

Table 1. Percentage of *Leucojum aestivum* L. leaf explants surviving each grade of the three factors

Grade	Leaf explants surviving (%)		
	Factor A	Factor B	Factor C
0	39.20	42.00	31.58
1	14.28	28.52	28.40
2	38.89	48.37	56.69
3	49.06	13.19	15.24
4	82.88	43.10	11.96

Table 2. Influence of factors A, B, and C on the sterilization efficiency in leaf explants of *Leucojum aestivum* L.

Variant	Factors ^a			Explants surviving ^b								
	Leaf explants inoculated in vitro			1 Repetition			2 Repetition			Total		
	A	B	C	1 Repetition	2 Repetition	Total	1 Repet.	Number	%	2 Repet.	Number	%
1	0	0	0	62	62	124	23	37.10	20	32.26	43	34.68
2	0	2	2	63	63	126	30	47.62	25	39.68	55	43.65
3	1	1	3	82	82	164	13	15.85	12	14.63	25	15.24
4	1	3	1	72	72	144	10	13.89	9	12.50	19	13.19
5	2	0	2	63	63	126	31	49.21	31	49.21	62	49.21
6	2	2	0	52	52	104	14	26.92	15	28.85	29	27.88
7	2	2	2	42	42	84	26	61.90	25	59.52	51	60.71
8	2	2	4	46	46	92	6	13.04	5	10.87	11	11.96
9	2	4	2	58	58	116	25	43.10	25	43.10	50	43.10
10	3	1	1	53	53	106	25	47.17	27	50.94	52	49.06
11	4	2	2	73	73	146	62	84.93	59	80.82	121	82.88

^aEach factor is tested in five grades:

Factor A: time from collection to in vitro culture. 0. Without storage (control); 1, 7 days; 2, 14 days; 3, 28 days; 4, 42 days.

Factor B: washing with running water before sterilization. 0. Without washing (control); 1, 4 h; 2, 16 h; 3, 24 h; 4, 48 h.

Factor C: time of sterilization 0.1% HgCl₂. 0, 30 s; 1, 1 min; 2, 2 min; 3, 4 min; 4, 6 min.^bThe results were reviewed after 4 weeks of cultivation.

Table 3. The smallest deviations (SD) between the variants of leaf explants of *Leucojum aestivum* L. proved by dispersion analysis

Variants	1 000	2 022	3 113	4 131	5 202	6 220	7 222	8 224	9 242	10 311	11 422
1 000	**	***	***	***	*	***	***	**	***	***	***
2 022		***	***	*	***	***	***	—	—	—	***
3 113		—	***	***	***	—	***	***	***	***	***
4 131		—	***	***	***	—	***	***	***	***	***
5 202			—	***	***	***	***	*	—	—	***
6 220				—	***	***	***	***	***	***	***
7 222					—	***	***	***	***	***	***
8 224						—	***	***	***	***	***
9 242							—	*	—	—	***
10 311								—	—	—	***

— < SD 5% = 5.453948; * > SD 5% = 5.453948; ** > SD 1% = 7.696055; *** > SD 0.1% = 10.99465

water, and with minimal exposure to treatment with the sterilizing agent. The percentage differences in the number of surviving explants are obvious. The optimal variants differ in leaf and bulb explants (Table 2).

The results of dispersion analysis show the difference in calculated probability between the variants. The differences between the variants are reliable (Table 3), which confirms the factor grading. The data give the following order for the 11 variants of leaf explants according to sterilization efficiency: 422 > 222 > 202 = 311 = 022 = 242 > 000 > 220 > 113 = 131 = 224 (> designates reliable difference; = designates lack of reliable difference).

This order shows the important influence of factor A for successful sterilization. The variant with maximum factor A is the best (42-day storage duration of the material). Variant 311 is less favorable than 222 due to less exposure to the sterilizing agent.

The 7-day storage of bulbs and leaves is crucial. The percentage of the explants surviving is lowest in variants 131 and 113, and 100% infection is observed in some cases. The longer exposure to $HgCl_2$ in variant 113 is not enough to compensate for the effect of the 7-day storage. Therefore successful bulb sterilization was significantly dependent on an increased length of time between collection and *in vitro* utilization.

Factor B most probably exerts loss influence on sterilization. The variants differing only in factor B follow the order: 222 > 202 > 242. Moderate leaf washing with running water is favorable. The difference between variant 222 and 202 is comparatively great (11.5%). Simmonds and Cummings (1976) and Visotskiy (1986), recommending washing the material before sterilization, explain the higher effect by the elimination of saprophytic microflora over the plant surface.

The order 222 > 220 > 224 is obtained after comparing the variants according to factor C. The tissue necrosis on a larger scale in variant 224 is due to long-lasting sterilization.

Based on the results of the dispersion analysis, the optimal conditions for sterilization of leaf explant are:

- length of time from collecting to in vitro inoculation – 42 days;
- washing with running water – 16h;
- exposure to 0.1% HgCl_2 – 2 min; 82.88% disinfected leaf explants are obtained in culture.

Sterilization of bulbs, stems, flower buds, and root tips encountered difficulties, and the percentage of disinfected explants was low.

2.2 In Vitro Development of Explants

In vitro regenerants and callus were recovered from bulb, leaf, and stem explants and there was no intensive callus formation from flower buds and root tips.

To prepare the bulb explants, the bulbs were cut in two parts along their widest diameter and each of these into eight radial pieces.



Fig. 2. Long-tube-shaped structures formed on a basal scale by direct organogenesis in *Leucojum aestivum* L. Four to 5 weeks after in vitro cultivation on MS1 or MS2 nutrient media based on MS with 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, and 0.5 mg/l nicotinic acid instead of vit. MS, 0.1 mg/l ascorbic acid and 45 g/l sucrose were added to MS1 and 30 g/l sucrose to MS2. Both media were supplemented with 1 mg/l NAA, 1 mg/l kinetin, and 1 mg/l BAP

Table 4. Comparative regeneration response of leaf and bulb explants from *Leucojum aestivum* L. cultivated on MS medium supplemented with 1 mg NAA, 1 mg/l BAP, and 1 mg/l kinetin

Explants	No. of inoculated explants	No. of explants with shoots	Total No. of regenerants	Average No. of regenerants per explant
Leaf sheaths	218	77 + 0.5	323	4.19
Tip of leaf	287	3 + 0.1	5	1.67
Young leaves	108	51 + 0.5	208	4.08
Bulb fragments	347	36 + 0.3	98	2.72

Four to 5 weeks after in vitro inoculation, three to six tube-shaped structures, some of them with curled periphery appeared between adjacent basal scales. They formed directly from the explant tissues (Fig. 2). The structures became dark green and differentiated into well-developed shoots, from the upper part of which the tips two leaves projected. Five to six shoots were formed on some of the explants. The lower part of the shoots became thicker and differentiated into a bulb. The explants excised from the innermost part of the main bulb and the bulblets formed the greatest number of regenerants, compared to all the other bulb explants – 2.92 and 2.38 regenerants per explant, respectively. Roots grew spontaneously or were induced by transferring the bulblets onto root induction medium. Treatment of the bulblets with low temperature (5°C) for a period of 4–6 weeks stimulated rhizogenesis in 85% of the regenerants obtained (Stanilova et al. 1994).

Explants from apical bulb scales showed exceptional development. White, thick, shapeless callus was observed on some of the bulb explants. Large calli (10 × 5 mm) were obtained in 25.6% of the inoculated scales and small calli in 41%. The explants from leaves and stems formed callus as separate white spots, and the explants from the ovaries formed larger callus under the surface of the medium.

Direct organogenesis was also observed from leaf and stem explants. The formation of shoots and bulblets was most active in the explants from leaf sheaths and from young leaves. An average of 4.19 and 4.08 regenerants per explant was obtained, respectively. The tips of the leaves exhibited the lowest regeneration activity, with an average of 1.67 regenerants per explant (Table 4). This is due to the difference in the extent of differentiation of the various parts of the leaves. The leaf sheaths and the young leaves are less differentiated than the tip parts, and possess better regeneration ability.

Regardless of the orientation of the leaf explant towards the regeneration medium, callus and organ formation always appeared at the cut towards the plant basis. The developmental pattern of the leaf explants, appeared to be highly dependent on their orientation towards the regeneration medium: callus formed when the explant cut towards the plant basis was submerged in the nutrient medium (8.06% of the inculcated explants developed); direct

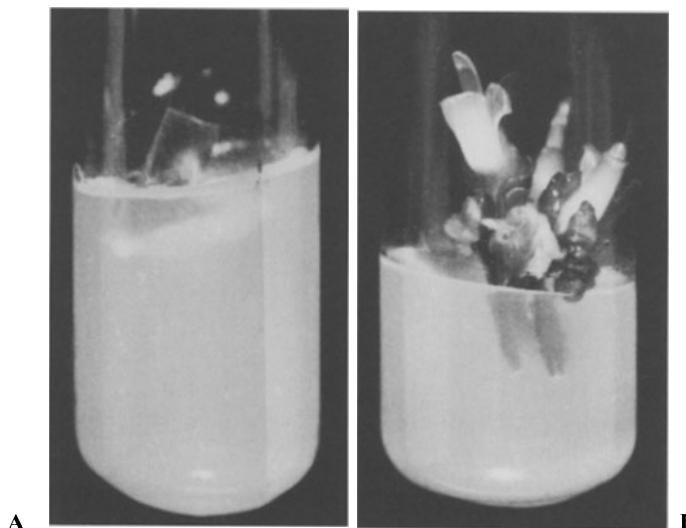


Fig. 3A,B. Developmental pathways of leaf explants of *Leucojum aestivum* L. depending on their orientation towards the regeneration media (MS1 or MS2 nutrient media based on MS with 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, and 0.5 mg/l nicotinic acid instead of vit. MS; 0.1 mg/l ascorbic acid and 45 g/l sucrose were added to MS1 and 30 g/l sucrose to MS2. Both media were supplemented with 1 mg/l NAA, 1 mg/l kinetin, and 1 mg/l BAP). **A** Callus on a leaf explant with its basal cut under the medium surface. **B** Shoots recovered via direct organogenesis from the basal cut of a leaf explant oriented upwards: **A** and **B** 8 weeks after cultivation during period of physiological activity

organogenesis was induced when the explant cut towards the plant basis was facing upwards (60% of the explants developed); both direct organogenesis and callus appeared on explants lying horizontally on the culture medium (71.43%) (Fig. 3). Shoots also developed at the periphery of the stem explants (Fig. 4), where the chlorenchyma is situated (Christova 1989). Such preferably orientated regeneration is probably due to the uneven distribution of endogenous hormones in the explant.

The in vitro regenerated plants possessed bulblets with a diameter of 5–17 mm, two to five dark green leaves with a length up to 12 cm and two to ten white or pale green roots which were ca. 9 cm long (Fig. 5; Stanilova et al. 1994).

Shoots and an insufficient amount of callus were obtained also from leaf fragments of seedlings and plants regenerated in vitro. However, these explants formed fewer bulblets (one to three) compared with leaf explants from donor plants (four to five) collected in the field. The lower green part of the leaves up to 10 cm above the bulb and the central and inner parts of the bulb were found to be the most suitable initial material for in vitro cultivation and plantlet regeneration. The high regeneration ability of these leaf and bulb explants is related to the small, densely packed meristematic cells possessing higher proliferation ability.

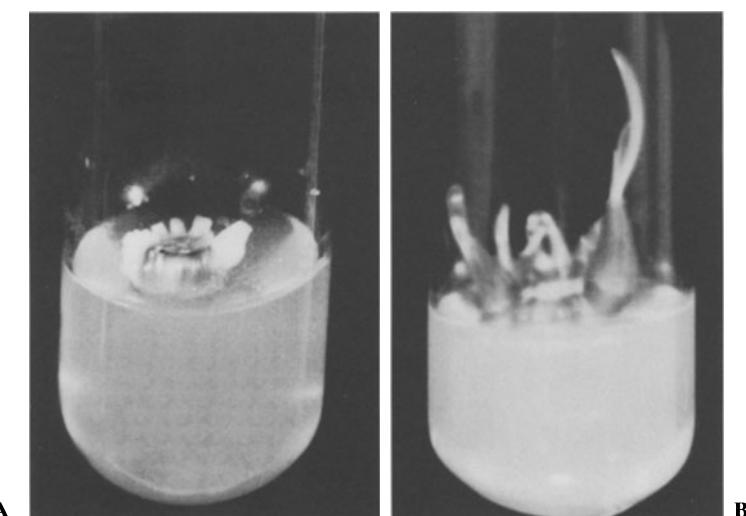


Fig. 4A,B. Consecutive development of shoots of 8 (A) and 16 (B) weeks of cultivation. Direct organogenesis on the periphery of a stem explant of *Leucojum aestivum* L. is achieved on MS1 or MS2 nutrient media based on MS with 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, and 0.5 mg/l nicotinic acid instead of vit. MS: 0.1 mg/l ascorbic acid and 45 g/l sucrose were added to MS1 and 30 g/l sucrose to MS2. Both media were supplemented with 1 mg/l NAA, 1 mg/l kinetin, and 1 mg/l BAP

Although the bulbs are available during the whole year, leaves are recommended to be used for the following reasons:

- the regeneration ability of leaf explants is much higher;
- the plants are not extirpated;
- leaf infection is considerably lower than in the bulb;
- the leaves are available during a period of physiological activity, and therefore there is no danger of stagnation in their in vitro development.

2.3 Nutrient Media and Cultivation Conditions

After preliminary testing of a number of nutrient media based on Linsmaier and Skoog (1965), Murashige and Skoog (1962), and Gamborg et al. (1968), supplemented with different quantities of NAA alone or in combination with kinetin, BAP, or DROPP [N-phenyl NI-(1,2,3-triadizolyl-carbamide)], finally several nutrient media were included in the experiment. They differed in the kind and ratio of their additives. MS medium supplemented with 1.1 mg/l 2,4-D (dichlorophenoxyacetic acid) was used for callus formation. Direct organogenesis was induced on MS1 and MS2 media based on MS with 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, and 0.5 mg/l nicotinic acid instead of vit MS; 0.1 mg/l ascorbic acid and 45 g/l sucrose were added to MS1 and 30 g/l sucrose to MS2. Both media were supple-

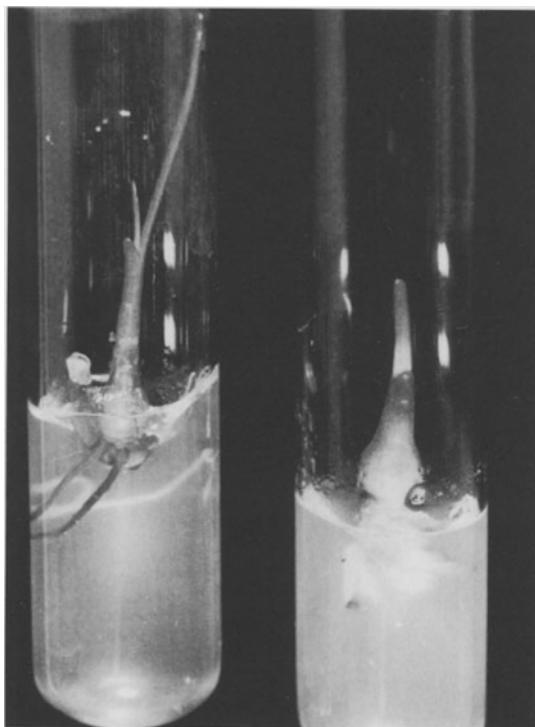


Fig. 5. Well-developed regenerants in *Leucojum aestivum* L. with a bulb, leaves, and roots, formed on a scale attached to the bulb basis after 5–6 months of cultivation during physiological activity. Rooting was achieved after 10 days on MS2 medium with a half reduced sucrose quantity (15 g/l), 0.1 mg/l NAA, 0.1 mg/l kinetin, 0.1 mg/l BAP and 5 g/l agar at 21 °C, and pretreatment at 5 °C for a period of 4–6 weeks

mented with 1 mg/l NAA, 1 mg/l kinetin and 1 mg/l BAP, 7 g/l agar, pH 5.8. MS2 medium with a half sucrose quantity (15 g/l), 0.1 mg/l NAA, 0.1 mg/l kinetin, 0.1 mg/l BAP, and 5 g/l agar, pH 5.8, was most favorable for root induction.

Interestingly, some explants exposed to identical cultivation conditions developed differently. For instance, callus, shoot, or very intensive root formation were observed on certain explants cultured on organ induction medium. This developmental pattern of explants differs from the common mechanisms of in vitro growth regulated by the quantitative interactions between phytohormones, especially auxins, cytokinins, and other metabolites (Skoog and Miller 1957).

Seeds grown on MS1 medium developed into seedlings with well-shaped white bulblets, and MS medium supplemented with 0.1 mg/l ascorbic acid, 0.5 mg/l NAA, 0.1 mg/l kinetin, 1 mg/l abscisic acid, and 7 g/l agar produced seedlings with a less well-formed bulb (Fig. 6). The difference is most probably due to the greater sucrose quantity in the former medium, favoring the deposit

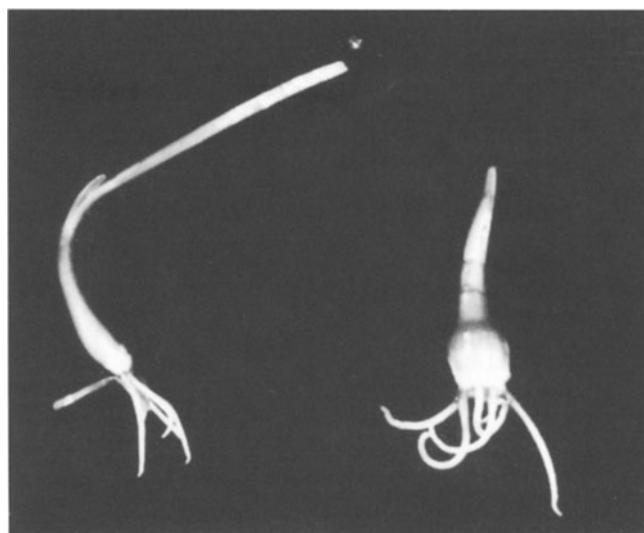


Fig. 6. Three to 4-week-old seedlings with less well formed bulbil grown on MS medium supplemented with 0.1 mg/l ascorbic acid, 0.5 mg/l NAA, 0.1 mg/l kinetin, 1 mg/l abscisic acid, and with a well-shaped bulb part grown on MS1 media based on MS with 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, and 0.5 mg/l nicotinic acid instead of vit. MS, 0.1 mg/l ascorbic acid, 1 mg/l NAA, 1 mg/l kinetin, 1 mg/l BAP, and 45 g/l sucrose

of reserve substances in the bulb, as well as to the presence of abscisic acid and auxins in the latter.

The clearly expressed period of physiological dormancy (July-October) was preserved during in vitro culture. Explant development always began in the first cultivation week during the period of vegetation activity: The explants did not change until October even when the experiments started at the end of June. Treatment with continuous darkness and low temperature (5°C) for 1 month was not able to interrupt the physiological summer dormancy of the explants and of the small shoots already formed.

Seed germination also exhibited season dependence. Regenerant and seedling rooting was mostly activated by the beginning of the period of physiological development. Pretreatment at 5°C for a period of 4–6 weeks stimulated rhizogenesis, which appeared up to 10 days after consequent cultivation on root induction medium at 21°C.

Similar to some other bulb species (Yeoman 1986), in vitro cultivation of *L. aestivum* was performed at a comparatively low temperature (21°C), 16-h photoperiod, and 1500 lx light intensity.

2.4 Cytological Analysis

A total of 104 plants regenerated in vitro (one root per plant) were cytologically analyzed. Investigations showed that all regenerated plants possessed a

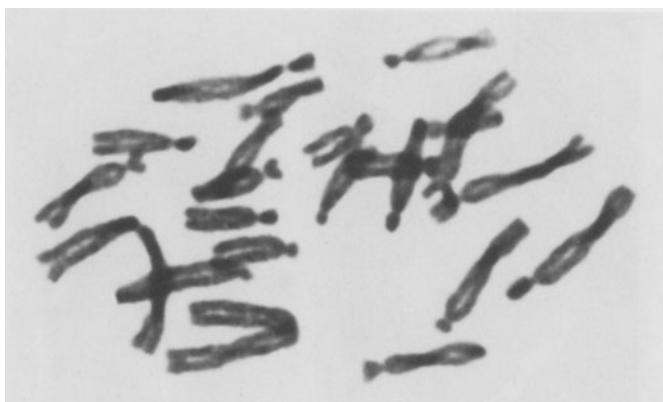


Fig. 7. A normal diploid number of chromosomes ($2n = 22$) in a root tip squash preparation in an in vitro-regenerated plant of *Leucojum aestivum* L.

normal diploid chromosome number ($2n = 22$; Fig. 7). Only single cells of some regenerants possess smaller chromosome numbers, 12 and 16. This difference is probably caused by the influence of exogenous phytohormones in the nutrient medium (Stanilova et al. 1994).

3 Summary and Conclusions

An effective system for in vitro micropropagation via direct organogenesis of the endangered and medicinally valued *Leucojum aestivum* L. has been developed. The initial material suitable for explants for in vitro micropropagation is distinguished by comparatively low infection and high ability for direct organogenesis. These are leaf sheaths, young leaves, and the central part of the bulb. Although the bulbs are available throughout the whole year, leaves are preferred for in vitro micropropagation as they possess the highest regeneration ability. They can be used without destroying the plant and are available for a comparatively long period of time at a stage of physiological activity. Moreover, the leaves are less contaminated than the bulbs.

Theoretically, 631 plants can be obtained from a single plant of *Leucojum aestivum* in 1 year using the described in vitro method (total number of cultivated explants from a single plant x average number of regenerants per explant). Under natural conditions, about 10–20 bulbs are formed from a single bulb by vegetative propagation over a period of 12–15 years. This could be a promising approach for the conservation of this endangered plant species and for providing plant material for industrial extraction of the galanthamine alkaloid used in medicine production.

4 Protocol

4.1 Sterilization of Initial Plant Material and Explant Preparation

Forty-two days after the leaves of *Leucojum aestivum* L. are collected, they are washed with water and detergent, cut in 7–8-long pieces convenient for surface sterilization, and rinsed for 16h under running water. The plant material is immersed for 1 min in 70% (v/v) ethyl alcohol and then sterilized in 0.1% HgCl for 2 min. Before inoculation, the leaves are cut into pieces of 0.5–1 cm and laid horizontally on the nutrient medium.

4.2 Nutrient Media and Culture Conditions

MS1 and MS2 media based on Murashige and Skoog (1962) medium with 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, and 0.5 mg/l nicotinic acid instead of vit MS are used; 0.1 mg/l ascorbic acid and 45 mg/l sucrose are added to MS1, and 30 g/l sucrose to MS2. Both media, supplemented with 1 mg/l NAA, 1 mg/l kinetin, 1 mg/l BAP, and 7 g/l agar are most suitable for the induction of direct organogenesis in young leaves and leaf sheaths (green leaves up to 10 cm above the bulb) of *Leucojum aestivum* L. MS medium with half sucrose (15 g/l), 0.1 mg/l NAA, 0.1 mg/l kinetin, and 0.1 mg/l BAP is applied for further growth of the regenerants and rhizogenesis. To promote root formation, the newly formed bulbules are pretreated at low temperature (5°C) for a period of 4–6 weeks.

The in vitro cultivation is performed in phytostatic chambers at 21°C, 16-h photoperiod, and 1500 lx light intensity.

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I.14 Micropropagation of *Mammillaria* Species (Cactaceae)

A. RUBLUO¹

1 Introduction

The genus *Mammillaria*, the largest in the Cactaceae family, constitutes one of the most popular groups of cacti. This popularity lies basically in the huge variability of the genus, in its forms, color of flowers, its small size, and relative ease of culture and maintenance, all of which make it a horticultural favorite. Unfortunately, these factors mean that many species of the group are threatened with extinction. The following sections present a brief description of this genus.

1.1 Botanical Aspects

Taxonomic aspects mentioned in this chapter conform with the Buxbaum system, which is based on morphological characters and phylogeny (Bravo 1978). The genus *Mammillaria* was named by Haworth in 1812 and is considered to be of recent evolution (Bravo 1978). Its name is derived from *mamilla* which means little tubercle.

Mammillaria presents dimorphic areoles, those that generate the spines, and the floral areoles on the axis of the tubercle. Spines can be central or radial, variable in number, form, size, and color. Flowers are small (10–25 mm in length and 15–50 mm in diameter) but conspicuous; their color can be from white to bright red, orange-red, pink, purple, or yellow; campanulate or tubular, solitary, in groups of two to three, but more usually forming a crown near the top of the plant.

Mammillaria species can be small single plants (5–24 cm in length) or multiple-headed, but can also be cespitose, forming small clusters, to large clumps over 1 m in diameter. Fruits are usually of a red color, bearing a large number (10–150) of small (1–2-mm) black or brown seeds per fruit.

1.2 Distribution and Importance

Most of the species in this genus have their center of genetic diversity in Mexico, growing from the coast to the mountains (elevation 3000 m.a.s.l.), but

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mostly in the arid and semiarid regions. Conservationist authorities like the International Union for Conservation of Nature (IUCN 1985); the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Hunt 1992), and the Mexican Ministry of Urban Development and Ecology (SEDESOL 1994) consider the genus *Mammillaria* a severely threatened group, particularly because of its high endemism. Black market, illegal gathering of plants from the wild, and destruction of habitat play a negative role in the losses in this genus (Oldfield 1985; Rubluo et al. 1993).

The potential of some *Mammillaria* species for medical purposes (hallucinogens) has been recognized. *M. craigii*, *M. grahamii*, and *M. senilis* have long been used by the Tarahumara Indians of Chihuahua Mexico (Evans and Hofmann 1979); moreover, an industrial potential has been claimed for *M. huitzilopochtli* (Rubluo et al. 1990).

1.3 Conventional Propagation and Need for In Vitro Culture

Mammillaria species that form side shoots are good candidates for vegetative propagation, which is accomplished by careful cutting at the base of the shoot near the mother plant. However, many species do not form lateral branches; in consequence, propagation by cuttings for these species is difficult, so that most mammillarias are propagated from seeds. Propagation through seeds should be performed after the seeds have been stored for several months (at least 3–6 months, J.S. Reyes pers. comm.) in dry conditions and cold temperatures (8–12°C) or even at room temperature. Fearn and Pearcy (1979) provided good tips for successful cultivation of *Mammillaria* species from seed.

Conventional practices are insufficient to fulfill commercial demands, particularly for those species with low germination rates, slow rates of growth, and slight or no lateral branching. In vitro propagation is an alternative that can offer strategies not only for recovering nearly extinct species (Rubluo et al. 1993), but also for providing opportunities for the commercialization of rare and endangered species (Rubluo et al. 1992) and even for maintaining and increasing the genetic diversity of otherwise endangered mammillarias (Rubluo et al. 1994).

Many *Mammillaria* are considered threatened with extinction and consequently, commercialization is restricted. A CITES resolution (Anonymous 1994) in support of the legal commerce of endangered species, provided artificial propagation is undertaken, will encourage concentrating on *Mammillaria*, and particularly on those species that are endangered. This illustrates the importance that in vitro culture techniques will have in the near future in the commercialization of rare plants, including *Mammillaria*.

2 Review of In Vitro Studies/Micropagation (Table 1)

First in vitro culture work in *Mammillaria* was reported by Minocha and Mehra (1974), and *Mammillaria woodsi* (Kolar et al. 1976) was the

Table 1. List of *Mammillaria* species cultured in vitro. (See also Hubstenberger et al. 1992)

Species	Explant	In vitro culture conditions ^a	In vitro response	Remarks	Reference
<i>M. prolifera</i>	Seedlings, vegetative buds, and floral organs	MS + kin (1–2 mg/l) + 2,4-D (10–20 mg/l) + CM (20–60%)	Callus		Minocha and Mehra (1974)
<i>M. woodii</i>	Stem sections (areoles) without secondary vascular bundles	MS + IAA + kin (2 mg/l) 25°C continuous light	Shoot regenerants with callus		Kolar et al. (1976)
<i>M. elongata</i>	Tubercles with spines trimmed	MS + 2ip (10 mg/l) + IAA (1 mg/l); 27°C 16 h photoperiod at 5 rlx	Shoot regenerants	Rooted in greenhouse without hormones	Johnson and Emino (1979)
<i>M. spaerica</i>					
<i>M. gracilis</i>					
<i>M. eichlamii</i>	Tubercle with spines trimmed	MS + 2ip (10 mg/l) + IBA (2 mg/l) 27°C 16 h photoperiod at 5 rlx	Poor callus Poor callus No response No response		Johnson and Emino (1979)
<i>M. spp.</i>					
<i>M. elongata</i>	Anther	Nitsch + 20% sucrose + 2,4-D (1–2 mg/l) + CM 1.5% + CH (200 mg/l) + kin (1–2 mg/l)	Callus		Cheema and Mehra (1981)
<i>M. glassii</i>	Axillary buds	Basal media not specified + BA (1 mg/l), no auxins	Multiple shoot formation		Starling and Dodds (1983)
<i>M. carmenae</i>	Stem sections from 2–3-year-old plants	MS + (1 mg/l) NAA + BA (2 mg/l)	Clusters of shoots		Výskot and Jara (1984)
<i>M. prolifera</i>	Stem section from 2–3-year-old plants	MS + BA + NAA (0.5 – 1 mg/l) 26°C ± 2.16 h photoperiod, 2000 lx	Clusters of shoots		Výskot and Jara (1984)
<i>M. gummifera</i>	Stem sections	MS + auxins + cytokinins (not specified)	Callus		Ault and Blackmon (1985)
21 different <i>Mammillaria</i> species	Seedlings	MS + 2,4-D + BA; 25°C 16-h photoperiod, 2500 lx	Callus and shoots		Damiano et al. (1986)
				Rooting in basal media, no growth regulation transferred successfully to soil	

Table 1. Continued

Species	Explant	In vitro culture conditions ^a	In vitro response	Remarks	Reference
<i>M. san-angelensis</i>	Stem sections	MS + BA (0.1 mg/l) or MS + BA (0.1 mg/l) + NAA (0.01 mg/l) or MS + BA (1 mg/l) + NAA (0.01 mg/l)	Multiple shoots Without callus		Martínez-Vázquez and Rubluo (1989)
<i>M. wrightii</i>	Shoot tips	L ₂ + several combinations of Picloram, zea and constant 20 °C ± 1 150 µmol/m ²	Multiple shoots	Rooting in hormone-free MS or NAA or IBA growth in greenhouse to flowering	Clayton et al. (1990)
<i>M. spp.</i>	Unfertilized ovules with placenta	MS + BA (2.25 mg/l) + IAA (0.18 mg/l); subcultured to MS + BA (0.225 mg/l) + IAA (2 mg/l)	Callus and embryonic primordia	Rooted in MS + IAA (1.8 mg/l) + kin (0.011 mg/l) reported somaclonal variation, rooting in hormone-free MS	Corneau et al. (1990)
<i>M. gracilis</i>	Lateral buds	MS + BA (2 mg/l)	Shoots without callus		Fay and Grattan (1992)
<i>M. spp.</i>	Lateral buds	MS + BA (2 mg/l)	Shoots with callus		
<i>M. albinaata</i>	Lateral buds	Not specified	Success on micropropagation		
<i>M. lasiacantha</i>	Lateral buds	Not specified	Success on micropropagation		
<i>M. mammillaris</i>	Seeds	Not specified	Success on micropropagation		
<i>M. nana</i>	Lateral buds	Not specified	Success on micropropagation		
<i>M. parkinsonii</i>	Seeds	Not specified	Success on micropropagation		
<i>M. solisoides</i>	Seeds	Not specified	Success on micropropagation		
<i>M. thersiae</i>	Lateral buds	Not specified	Success on micropropagation		
<i>M. viperina</i>	Lateral buds	MS + BA (1 mg/l) 27 °C ± 1, 16-h photoperiod, 3.2 W/m ² ,	Callus and shoots		
<i>M. huizilopochtli</i>	Stem section	8 h darkness	Multiple shoots without callus		
<i>M. san-angelensis</i>	Stem sections	MS + BA (0.1 mg/l) 27 °C ± 1, 16-h photoperiod, 3.2 W/m ² , 8 h darkness	Callus and shoots restored to the site and producing fertile seeds		Rubluo et al. (1993)

^a Basal media + growth regulators at the indicated concentrations + incubation conditions.

first reported cactus propagated by tissue culture. Nowadays, over 40 species of *Mammillaria* have been cultivated in vitro. Table 1 shows these efforts.

The aim of most of the studies had been with a view to plant regeneration, and in vitro seed germination has also been attempted with success (Table 1). However, research has also been conducted for other purposes. Minocha and Mehra (1974) used callus induction in *M. prolifera* for nutritional and morphogenetic investigations. Cheema and Mehra (1981) cultivated anthers of *M. elongata* in order to produce haploid plants. Rubluo et al. (1990) used *M. huitzilopochtli* callus as a possible source of betalains. Gratton and Fay used tissue culture as an effective way to eliminate fungal and bacterial soft rots from *Mammillaria* sp. (cited by Fay and Gratton 1992).

Somaclonal variation has been reported by Corneau et al. (1990) in *Mammillaria* spp; however, these authors do not state if the changes were genetically stable or epigenetic.

3 Micropropagation Procedures

Of the three processes for in vitro plant regeneration: (1) production of shoots from preexisting meristems, (2) generation of adventitious shoots through callus formation and (3) direct somatic embryogenesis or indirect (callus-cell suspension culture-mediated), the genus *Mammillaria* had benefited from the first two; however, micropropagation through somatic embryos in Cactaceae has been realized in at least two instances: *Ariocarpus retusus* (Stuppy and Nagl 1992) and *Aztekium ritteri* (Rodríguez-Garay and Rubluo 1992). Moreover, it was known that species that naturally show polyembryony are prone to produce embryogenic callus (Tisseratt et al. 1979). As early as 1956, Tiagi (cited by Tisserat et al. 1979) described polyembryony in *M. tenuis*, paving the way to attempt embryogenic callus in *Mammillaria*.

Mammillaria is a genus that needs all three kinds of micropropagation, because many of its species are on the verge of extinction and because of its commercial use.

3.1 Choice of Explants and In Vitro Culture Initiation

A clear relationship between the selected explants for in vitro culture and success in micropropagation in mammillarias was reported (Johnson and Emino 1979; Martínez-Vázquez and Rubluo 1989), most of the authors using meristematic tissue: lateral buds or parts of seedlings that include the areoles (Table 1). This was due to the primary interest in having genetically stable individuals as well as attaining mass propagation, in the absence of callus (Mauseth 1979; Martínez-Vázquez and Rubluo 1989; Hubstenberger et al. 1992). However, it must be mentioned that the use of tissue that includes

meristems does not guarantee the avoidance of callus (Johnson and Emino 1979; Viskot and Jara 1984), mostly due to the growth regulator balance in the culture media. Some other explants were also used; Kolar et al. (1976) cultured pith from *M. woodsii*, obtaining plant regeneration, but via callus; however, the same group (Havel and Kolar 1983), using similar procedures (but different media and growth regulators), was unable to obtain plant regeneration, obtaining only callus, from *M. prolifera*.

The type of explant used depends on the availability and the aim of the research. Rubluo et al. (1993) reported a strategy for the recovery of severely endangered cacti and recommended the use of seedling tissue from seeds germinated in vitro. In this way, contamination can be avoided, particularly with bacteria, which in *Mammillaria* is a major problem when explants are obtained from adult plants, due basically to the high number of fine spines that make normal disinfection procedures difficult (Johnson and Emino 1979; Havel and Kolar 1984; Fay and Gratton 1992). Moreover, when scarcity of material is a problem, preliminary experiments should be done with closely related species (Martínez-Vázquez and Rubluo 1989; Rubluo et al. 1993). When possible, the use of seeds to attain explants is recommended; it usually takes 2–6 months for seedlings 5–10 mm long to be ready to be used as a source of explants.

When explants from adult plants are used, the decontamination process may be stronger, including several surface sterilization (Hubstenberger et al. 1992).

3.2 Culture Media and Growth Regulators

Murashige and Skoog (1962) basal nutrient media (MS) have been used in Cactaceae and the *Mammillaria* (Hubstenberger et al. 1992; Fay and Gratton 1992). Even though MS has proved to be adequate in most cases, research is still needed with regard to the nutritional requirements of the Cactaceae to be more efficient in obtaining regeneration in *in vitro* cultures. For instance, Clayton et al. (1990) tested several species of Cactaceae, including *M. wrightii*, on five different basal media for axillary shoot proliferation. They found that L2 gave the best results, while MS, SH, and B5 were quite similar and only half-strength MS described by Gladfelter and Phillips (Hubstenberger et al. 1992) performed poorly; however, these authors pointed out that L2 not only has a high total major and minor salt content compared to the other media, but also magnesium and calcium ions, which are important in the nutrition of the Cactaceae. The low organic matter content, as well as the abundance of calcium carbonate in the soils where mammillarias grow should be taken into account when selecting a culture medium. Most of the media include calcium in their formulas as calcium chloride at different concentrations; for calcium, B5 includes an ionic composition of 2 mEq/l and MS 6 mEq/l, whereas L2 has 8.2 mEq/l ionic calcium (George and Sherrington 1984). These data are in line with the results of Clayton et al. (1990).

Among *Mammillaria* species, the necessity for an adequate balance in growth regulators seems to be very precise and highly specific to each of those studied (Johnson and Emino 1979); however, some general outlines can be drawn: the presence of an exogenous cytokinin is generally always required in order to obtain shoot proliferation in vitro (Hubstenberger et al. 1992). In contrast, auxins are not strictly necessary, and they may be an unwanted factor because they usually induce callus production, thus increasing the chance of genetic instability; moreover, Hubstenberger et al. (1992) proposed that cacti appear to have the capacity to produce excess auxin in vitro, so most authors use low auxin concentrations or try to avoid it (Table 1). Moderate concentrations of cytokinins are required for micropagation of *Mammillaria*; the most frequently employed are BA or 2ip (Table 1).

The usual combination of auxins and cytokinins in *Mammillaria* could lead to plant regeneration, but is associated with callus production (Kolar et al. 1976; Johnson and Emino 1979; Viskot and Jara 1984). The use of cytokinins alone induces shoot proliferation, usually without the production of callus (Starling and Dodds 1983; Martínez-Vázquez and Rubluo 1989; Hubstenberger et al. 1992). Investigators interested in this field should consider these general observations.

3.3 Other Factors

As a member of the Cactaceae family, *Mammillaria* shares many of the particular physiological characteristics of this group, which should be taken into account when envisaging in vitro propagation. These characteristics are mainly: (1) the condition of being crassulacean acid metabolism plants, (2) the water status, very low under natural conditions, contrasting with the in vitro culture vessels, in which humidity is close to 100%, (3) temperature of growth, which in the natural environment presents a wide range: from subzero to +50°C, and (4) irradiance, that in the growing areas of these species reaches very high values.

So far no effort has been made towards the evaluation of the effect or consequences of crassulacean acid metabolism in vitro (acid accumulation and change of pH). Concerning the humidity content and growth temperature, the cacti have proven the capacity to respond under in vitro culture conditions because of these two factors: 100% humidity seems not to affect the morphogenetic capacity of cultured explants, and a good response is generally achieved when the explants are incubated at a range of 20–30°C constant temperature. The role of irradiance also has been ignored in research of in vitro culture of Cactaceae, and general acceptance of values around 3 W/m² are usually reported as beneficial for plant regeneration. However, Hubstenberger et al. (1992) report the beneficial use of very high, for in vitro culture unusual, levels of irradiance (up to 15 W/m²); with this procedure they avoid vitrification of the shoots. These conditions are convenient because any standard plant tissue culture laboratory can achieve them.

Johnson and Emino (1979), working with several *Mammillaria* species under similar experimental conditions, found very different responses: from 100% success in plant regeneration (*M. elongata*) to absolute failure (*M. eichlamii*). In our laboratory, when similar strategies were used for *M. sanguineous* and *M. huitzilopochtli*, the response was distinct for every species. While in the first, under certain treatment successful mass propagation was attained (21–35 shoots per explant) without any callus formation (Fig. 1a; Martínez-Vázquez and Rubluo 1989), in *M. huitzilopochtli* cultures, callus was always present (Fig. 1b) and a low yield of shoots obtained (all vitrified), although medium and growth regulators were the same (Arriaga et al., in prep.). From these observations we conclude that the genetic factor plays an important role in the morphogenetic expression of in vitro cultured *Mammillaria*.

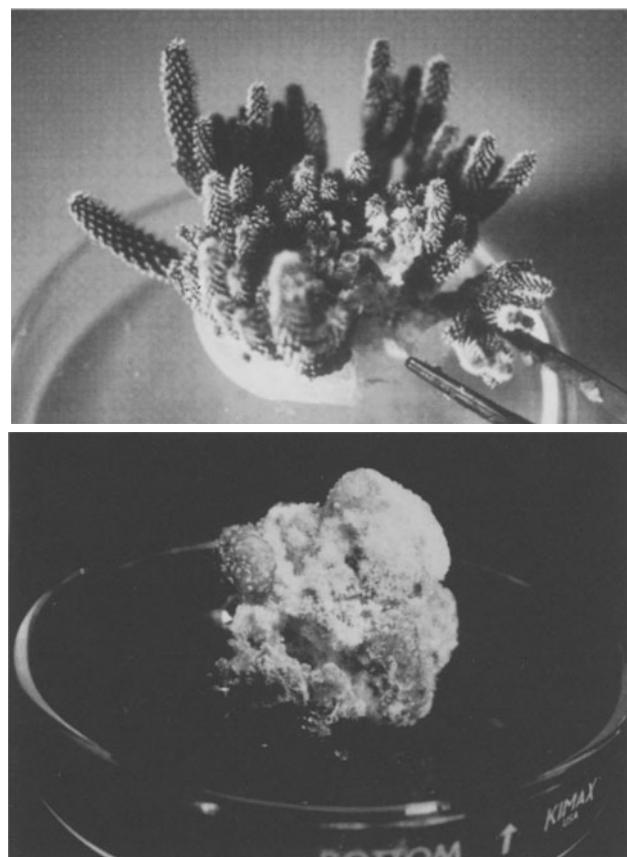


Fig. 1a,b. Differential responses of in vitro culture of two *Mammillaria* species cultivated under the same conditions. (MS + 1 BA + 0.01 NAA, mg/l; 16 h light (3.2 W/m²) 8 h darkness. **a** *M. sanguineous*. The multiple shoot formation was attained without any callus. **b** *M. huitzilopochtli*. Callus was always present and scarce formation of vitrified shoots

3.4 Rooting and Reestablishment

Rooting in vitro-derived mammillarias in general is considered an easy task, and Hubstenberger et al. (1992) report eight *Mammillaria* species, of which six rooted spontaneously without auxin, while the others were rooted after treatments with IBA or IAA; however, rooting efficiency was not recorded.

Rubluo et al. (1993) reported that in *M. san-angelensis* root systems are easily obtained in individualized shoots subcultured in hormone-free MS medium (Fig. 2a); 100% success in developing roots was obtained. Similar results were found for *M. haageana*, and survival into the greenhouse was also 100% for both species. However, the same authors' efforts to root *M. huitzilopochtli* spontaneously were unsuccessful, and only after treatments with auxins (best result with IAA 0.01 mg/l) was a comparatively poor response obtained (one to two roots per shoot; 0.2–0.4 cm length). Furthermore, some of these roots were not directly connected with the shoots and a callus coat was formed from which the roots emerged. In some other experiments (with the same treat-

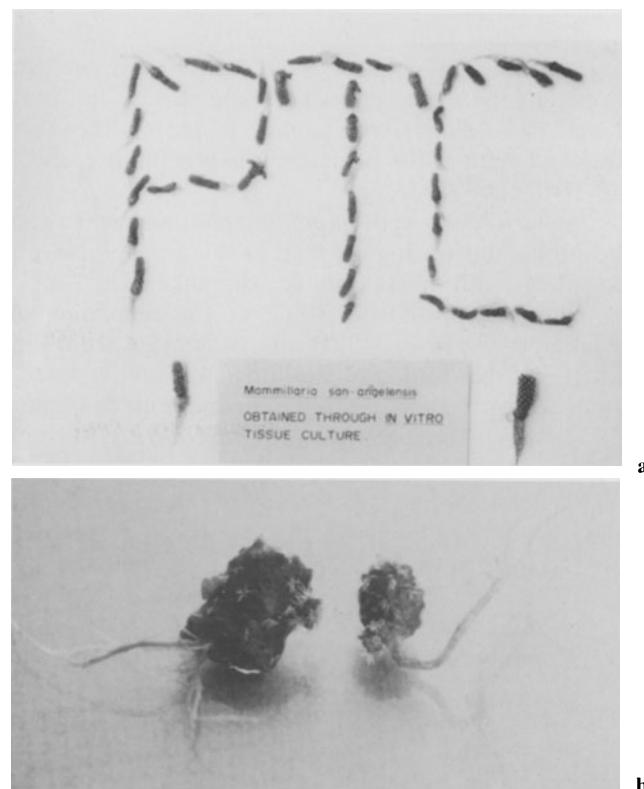


Fig. 2a,b. Root development on micropropagated shoots of two *Mammillaria* species (6–12 months of age). **a** *M. san-angelensis* individualized and subcultured on hormone-free MS. **b** *M. huitzilopochtli* individualized and subcultured on MS + 0.01 mg/l IAA

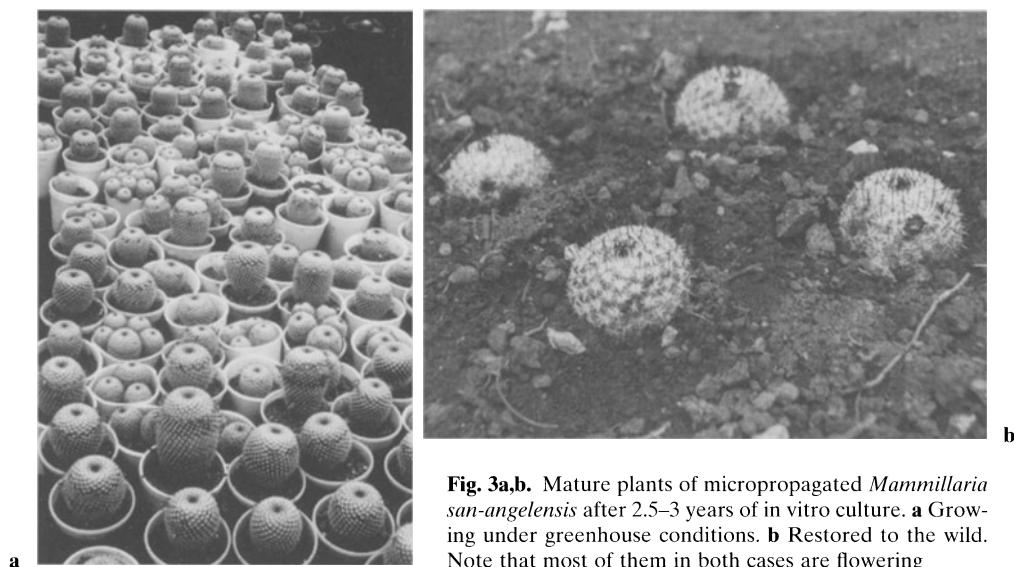


Fig. 3a,b. Mature plants of micropropagated *Mammillaria san-angelensis* after 2.5–3 years of in vitro culture. **a** Growing under greenhouse conditions. **b** Restored to the wild. Note that most of them in both cases are flowering

ment), callus did not occur, and the roots formed directly but still with a poor number (one to two roots per explant, Fig. 2b), but with an improvement in length: 0.5–1.5 cm (Arriaga et al., in prep.). These results obviously affect the establishment of the plant into the greenhouse, and values of survival around 50–60% were recorded.

In vitro-derived *Mammillaria* plantlets with a good root system do present no problem on being adapted to the greenhouse, provided all the usual care for plants with this origin had been taken.

Sterilized soil from some of the recommended mixtures (Fearn and Pearcey 1979; Reyes 1994), or even better, from the plants' natural environment, can be used, and the little plantlets watered with Hoagland solution (half-strength) or water, but avoiding both desiccation and overflooding.

In our experience with *M. san-angelensis*, full development of plants, from explant in vitro inoculation to flowering in the greenhouse or in the field (Fig. 3a,b), takes around 2.5–3 years, which is apparently less or about the same time as in vivo; however, this is difficult to say because lack of phenologic studies for this species does not permit comparison.

3.5 Commercialization of *Mammillaria* Through Biotechnology

The main importance of *Mammillaria* is its esthetic value as an ornamental and for this reason it has been heavily exploited in the wild. The solution for resolving this problem is to offer these plants to the legal market, but artificially produced instead of being collected from their environment. Thus mass propagation in a reasonable length of time and at a competitive cost must be realized. This can be achieved through in vitro culture technologies.

4 Summary and Conclusions

The genus *Mammillaria* is mostly endemic to Mexico, and an ornamental favorite; however, due to habitat destruction and overcollection, many of its species are endangered.

Artificial propagation of rare (or common, but valuable) species is considered a desirable way of reducing the pressure on wild populations. In vitro culture has proved to be an efficient method of mass propagation of this genus. Culture of preexisting meristems, adventitious shoots through callus formation, and somatic embryogenesis has been explored with various degrees of success. However, there are still problems in achieving successful micropropagation in a wide range of *Mammillaria* species. The genetic factor seems to be a limitation that can be overcome after the formulation of a specific medium, a proper environment, and an adequate balance of growth regulators.

Rooting in some species could be a problem that would impair economic viability. The possibility of restoration, through in vitro techniques, of a severely endangered *Mammillaria* to the wild, with production of flowers, fruits, and fertile seed development has been demonstrated.

5 Protocol

Lateral explants from young seedlings (from in vitro-germinated seeds) constitute the best source for micropropagation of *Mammillaria*. For multiple shoot proliferation, explants inoculated in MS and subject to one of three possible treatments: BA alone at 0.1 mg/l, BA 0.1 mg/l combined with NAA at 0.01 mg/l, and BA 1 mg/l or in combination with NAA 0.01 mg/l give the best results. Optimal incubation conditions are 3.2 W/m² 16h, 8h darkness, and 27°C constant temperature. Rooting can be easily achieved after subculturing individualized shoots in hormone-free MS or supplemented with auxins (e.g., IAA 0.01 mg/l). One month later, pot in the greenhouse (in their own soil) and protect from sunlight and desiccation.

This is a general protocol; however, genetic factors play a strong role and procedure should be adapted consequently.

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I.15 Micropropagation of *Mediocactus coccineus* S.D. (Yellow Pitaya)

R. INFANTE¹

1 Introduction

1.1 Botany, Distribution, and Importance

Pitayas are succulent rustic cacti, (Cactaceae family) originating from tropical America, and found in a wild state in Colombia, Venezuela, Brazil, Mexico, and Costa Rica. They are usually found from sea level up to 1800m (Britton and Rose 1963).

There are different cactus genera (*Mediocactus*, *Cereus*, *Hylocereus*, *Stenocereus*, *Selchicereus*, *Acanthocereus*) having edible fruits called pitaya, the spelling of which changes with different places: pitaya, pitajaya, pitalla, etc. The actual taxonomy of the group is not sufficiently clear, because of multiple renaming and reclassification. Nevertheless, there are two main known groups: yellow and red pitaya. The yellow pitaya (*Mediocactus coccineus* S.D. and *Cereus triangularis* Haw.) are the most important and widely cultivated.

The flower is perfect, white or pink, nearly 20cm long; it blossoms at night exhaling an aroma attractive to insects and bats that usually act as pollinators. After setting, the fruit grows, depending on temperature, for 4 to 8 months until maturity.

Yellow pitaya plants grow as climbers on trees or stones, or are procumbent when deprived of support; they possess strong green triangular stems with concave faces. They can grow freely for more than 6m, but when cultivated are pruned to maintain them below 2–2.5m. Flowers are white; fruits are yellow at maturity, up to 12cm long and the flesh is sweet and white, with little black seeds. *M. coccineus* is the most appreciated because of its flavor, and is best adapted to transport and storage among all the pitaya species.

Furthermore, the flowers of yellow pitaya could also be commercially exploited because they are rich in captine, a cardiotonic available to cardiologists who could substitute it for digitalis or similar treatments. There are no studies concerning the selection of genotypes rich in this product (Vasquez 1982).

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Few diseases have been reported affecting pitaya. Anthracnose on the fruit and stem caused by *Colletotrichum* sp. has been observed by Becerra-Ochoa (1988). Bacterial root and stem rot on poorly drained soils has also been reported.

Insect pests generally do not limit production, however, under monoculture conditions, problems associated with insects could be important. Larvae of the fruit fly (*Anatrephes* sp.) attack young fruit, making them unmarketable. The main phytosanitary problem observed in Colombia is caused by nematodes: *Meloidogyne incognita* race 2 and *Heterotylenchus dihystera* are the most prevalent (Rincon et al. 1989).

Yellow pitayas have been considered as a valid alternative to cultivation for some highlands of Colombia and Venezuela where at present they exist as monocultures of coffee. Local cooperatives and research institutes have been interested in recent years in the development of the culture of yellow pitayas; their studies mainly consider agronomic aspects, pest and disease control, storage, and marketing. The fruit has been appreciated in Japan and Europe, but the volumes shipped are still small because commercial cultivation has started only in the last few years.

1.2 Conventional Propagation

M. coccineus has been traditionally propagated asexually through cuttings. Propagation through seed is also practiced, but seedlings show increased genetic heterogeneity and the juvenile period is too long, up to 6 years (Becerra-Ochoa 1988).

Variety releases have not resulted from conventional breeding, but from selections chosen from seedling populations obtained from uncontrolled pollinations. Plants selected in nurseries as stock material are those showing the best agronomic characteristics and the best health.

The traditional propagation method of *M. coccineus* consists of the excision of cuttings 25–30 cm long from mature stems; afterwards cuttings are left in ventilated rooms to induce healing to reduce fungus attacks. Application of contact fungicides on the wound zone is usually recommended (Becerra-Ochoa 1988). After 1 week the cuttings are immersed in a root-inducing hydro-alcoholic solution composed of 1000 ppm naphthalenacetic acid (NAA) for 30 min. The cuttings are then placed in plastic bags containing a peat–sand–soil mixture and are maintained in a ventilated and partially shaded area and watered periodically until transplantation into the field (Figs. 1, 2).

Shimomura and Fujihara (1990) have further suggested soaking the apical end of the cuttings of *Hylocereus trigonus* in benzyladenine (BA) before transplanting to soil. This treatment increased the ratio of cuttings with sprouted buds, the number of sprouted buds, and the shoot length compared to the water control.

The same authors compared the effect of cutting length (7 and 15 cm) on rooting and budding capacity when treated apically with BA and at the base



1



2

Fig. 1. View of a nursery of conventionally propagated yellow pitaya as cuttings, at the Cauca Valley, Colombia

Fig. 2. Closer view of conventionally propagated yellow pitayas already prepared for transplantation to soil

with NAA. The number and length of BA-induced axillary shoots in the longer cuttings were higher than those in the shorter ones. In the 15-cm-long cuttings, increasing the soaking time from 5min to 24h resulted in a greater promotive effect of BA on shoot formation. BA applied before curing showed the same effect as that given after curing but caused necrosis of the tissue just under the cut surface.

1.3 Need for Micropagation and In Vitro Studies

There is a need to select genotypes showing positive traits: high and constant fruit production, short juvenile phase, and resistance to pests and diseases. The selection carried out so far has been unsystematic, and local endeavors have sometimes produced only a limited impact.

The first step to take for this recently domesticated species is the identification and characterization of many local selections, in order to reduce the confusion of domestic naming. Afterwards, the best genotypes should be tested under different culture conditions. In these two tasks, in vitro culture techniques could offer an efficient tool for achieving the objectives in less time than traditional propagation methods could allow.

The availability of a large number of selected clones of pitaya is necessary for the development of modern commercial plantations. In vitro culture could offer the way for mass propagation of these clones.

2 Micropagation

The first attempt to mass propagate cacti in vitro was made about 40 years ago (King 1957). Since then there have been numerous reports on the in vitro culture of various cacti and the subject has been recently reviewed (Hubstenberger et al. 1992; also see Chap. I.14, this Vol.). However, there is as yet only one publication on yellow pitaya (Infante 1992).

Tissue culture techniques can overcome certain limitations associated with conventional propagation of cacti (Clayton et al. 1990). Cactus micropagation through axillary branching has shown a high degree of phenotypic uniformity within clones (Ault and Blackmon 1987). In pitaya, as in all other cactus species, there are no studies comparing the field performance of in vitro- and traditionally propagated clones.

Our studies on yellow pitaya (Infante 1992) reporting the culture of seedlings on MS (Murashige and Skoog 1962) medium for callus induction, shoot proliferation, and the formation of somatic embryos are summarized below.

Micropropagation of pitaya has been obtained both by sowing sterile seeds picked from ripe fruits and dried for 2 weeks at room temperature and by culturing buds from in vivo plants. In both systems, the explants were surface sterilized by sodium hypochloride, 10% for 15 min for seeds and 5% for 10 min

for *in vivo* buds. The explants were rinsed several times afterwards with sterile tap water.

Once seeds have germinated on MS (Murashige and Skoog 1992) medium without growth regulators, the epicotyls (15–20 mm long), roots and cotyledons were removed and transferred to the proliferation medium. In the case of mature buds as explants, MS medium enriched with 0.6 μM BA and 0.6 μM IBA ensures a suitable initial shoot growth.

At the second subculture, shoots from both sources can be transferred to the same proliferation medium producing similar responses.

2.1 In Vitro Shoot Proliferation

Media tested for *in vitro* proliferation were based on MS mineral salts supplemented with 116.6 μM *myo*-inositol, 1.2 μM thiamine-HCl, 3% sucrose, and 0.7% Difco Bacto agar, pH adjusted to 5.7. Cultures were maintained in a growth chamber at $23 \pm 2^\circ\text{C}$ with 16/8 h light/dark regime, photon flux density (PFD) 30–40 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Pitaya explants seem to possess a high internal auxin level, because they can proliferate and form basal callus even on medium without growth regulators. Nevertheless, when 4.4 μM BA and NAA or IBA at 0.05–0.27 μM are added to the medium, the shoot proliferation rate is enhanced (Fig. 3). The highest proliferation rate obtained in a medium supplemented with BA and NAA was 6 shoots/initial after 6 weeks in culture. Under these conditions there is a lot of basal callus formation and sometimes it is possible to regenerate from it; furthermore, shoots are small, so it is important to induce their elongation before transfer to the root-inducing medium.

Shoot proliferation rate can be further enhanced if the apical shoot is removed (Table 1). This simple procedure limits the internal auxin level of explants, so that apical dominance is inhibited, basal budbreak is promoted, and a more effective cytokinin effect is attained. When decapitated shoots are cultured on the same medium reported before the shoot, the proliferation rate is 7.8 shoots/initial and shoot length increases to 40%, eliminating the need for a supplementary elongation of the shoots prior to rooting.

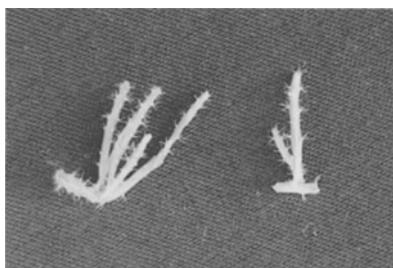


Fig. 3. Proliferating shoots of yellow pitaya after 8 weeks on an MS medium supplemented with 4.4 μM BA and 0.27 μM NAA

Table 1. Intact and decapitated shoots of yellow pitaya cultured on MS media with different NAA and BA concentrations. (After Infante 1992)

Growth regulators (μ M)		Proliferation rate (shoots/initial)		Shoot length (mm)	
BA	NAA	Intact	Decapitated	Intact	Decapitated
2.2	0.05	5.0 ab	6.9 b	12.0 ab	16.4 a
2.2	0.27	5.4 ab	7.7 a	12.7 a	14.9 a
2.2	0.54	4.1 bc	6.3 b	11.4 b	12.6 b
4.4	0.05	5.3 ab	6.7 b	13.3 a	15.8 a
4.4	0.27	6.0 a	7.8 a	11.1 b	14.0 ab
4.4	0.54	3.6 c	5.9 b	11.0 b	12.1 b

Means followed by the same letter in each column do not significantly differ at 5% level by Duncan's multiple range test.

2.2 Callus Culture and Somatic Embryogenesis

Highly friable callus could be easily induced from seedlings or proliferating shoots on media enriched with NAA and BA. It could be maintained through periodical transfers onto the same medium for more than 30 weeks without losing its morphogenic ability. Shoot induction from this callus requires a medium with a high BA content (4.4–8.8 μ M). In this condition callus becomes compact and green nodules are formed from which shoots develop through caulogenesis.

Somatic embryogenesis is attained when this callus is transferred to MS medium without growth regulators: after 4–5 weeks white globular-shaped clusters are formed on the surface. After 2 more weeks, some of these globular structures elongate and show an oval shape, and cotyledons become evident. At this moment the embryo-like structures are well defined, and can be transferred to a new hormone-free medium to induce germination. Somatic embryogenesis has also been obtained by transferring callus from a highly enriched NAA medium to an MS medium enriched with 17.6 μ M BA.

2.3 Rooting and Acclimatization

Due to the high auxin internal level of pitaya explants, it is easy to induce root formation. It is a common event to observe cultures on proliferation medium that form aerial roots borne from vegetative buds, when left for more than 2 months.

For the induction of a healthy root system an MS medium enriched with 0.1 μ M IBA is required. After 2 weeks on this medium several lateral roots are formed, capable of supporting the weaning stage.

Acclimatization is not particularly difficult in this species because the succulent structure and waxy surface of the plant is not highly susceptible to

water loss. Two weeks of acclimatization in the greenhouse prior to transferring the plants to plastic bags in the field protected by a plastic shading net are enough for a successful weaning.

The major difference seen in a tissue-cultured plant in contrast to a cutting-propagated one is the axillary branching which occurs higher up in the stem in the first case. This could be particularly important because the basal branching of the tissue-cultured plants could have an impact on the cultural practices applied to the plants.

2.4 Commercial Aspects

Rare and exotic fruit can be sold in European markets at high prices. Japan is also a potential market for pitaya, and imported 22 tons in 1990. These are very exacting markets, therefore, fruits should be excellent in external appearance and quality with regard to size and color (Kitagawa et al. 1990).

Amounts of imports are still low, because pitaya is not well known yet; nevertheless, consumption has been increasing in the last few years and it should continue to increase if growers can offer high quality fruit and solve some postharvest problems (Toro 1988). However, it should be considered that yellow pitaya is a marginal exotic fruit and volumes traded in the international market will always be small.

3 Summary and Conclusions

Yellow pitaya (*Mediocactus coccineus* S.D.) is a tropical cactus cultivated in tropical America for its excellent fruit. In the last few years there has been an increasing demand for it, nevertheless, high quality plants are not available in the producing countries.

Tissue culture could furnish a valid tool for mass propagation and conservation of selected clones, free of diseases. Furthermore, as pitaya is easily cultured in vitro, regeneration from callus could be induced to produce somaclonal variation, useful in future breeding programs.

It should be important to test the performance of in vitro-propagated plants, in the field and compare it with the traditionally propagated ones.

4 Protocol

1. Sterilize seeds in 10% sodium hypochloride for 15min, then sow on MS medium without growth regulators. Sterilize buds on 5% sodium hypochloride for 10min and transfer to MS medium enriched with $0.6\mu\text{M}$ BA and $0.6\mu\text{M}$ IBA.

2. Transfer epicotyls and shoots to shoot-inducing medium (MS 4.4 μ M BA and 0.27 μ M NAA). After one or two subcultures decapitate shoots (excision of 1–2 mm of the apical tip) and transfer to the same medium.
3. Transfer to the MS rooting medium enriched with 0.1 μ M IBA. After 2 weeks on this medium transfer to greenhouse.

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I.16 Micropagation of *Mussaenda* Cultivars

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1. Introduction

1.1 Botany, Distribution, and Importance

The genus *Mussaenda* (family Rubiaceae) consists of ornamental shrubs or small trees grown in the subtropical and tropical regions of the world for the one to many colorful, large petaloid sepals of scarlet, pink, picotee, or white (Fig. 1). According to Rosario (1984), the development of the *Mussaenda* cultivars is the greatest contribution from the Philippines to the field of ornamentals. Current cultivars were developed through a breeding program at the University of the Philippines at Los Baños (Rosario 1987). The cultivars were named after important women in Filipino history (Rosario 1984).

The inflorescence consists of a branched terminal cyme with many pubescent flowers which become pigmented and mature basipetally (Rosario 1984). Flowers are bisexual in structure, but become functionally unisexual as a result of heterostyly (Rosario 1984). The most characteristic feature of the genus is the one to many enlarged, lanceolate, pigmented calyx lobes or sepals (Fig. 1). The corolla is comprised of five cream-yellow to red-orange lanceolate lobes with a pubescent tube extended downwards from the center.

The current *Mussaenda* cultivars were developed during the 1940s and 1950s at the University of the Philippines at Los Baños. In the early 1900s, a mutant form of *M. philippica* A. Rich was found at the base of Mt. Tuntungin. This mutant, later to be called Doña Aurora, exhibited five extended white sepals instead of the single, extended sepal displayed by *M. philippica*. Doña Aurora was hybridized to *M. erythrophylla*, an African *Mussaenda* species which exhibits a single red sepal. The resulting progeny were self-pollinated and backcrossed to both parents (Rosario 1984). The progeny of these crosses later became the cultivars used today.

The genus *Mussaenda* comprises about 200 species of subtropical to tropical shrubs and trees native to tropical Africa, Southeast Asia, Australia and

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Fig. 1. Enlarged pigmented calyx lobes or sepals of *Mussaenda* Queen Sirikit

the Pacific Islands (Bailey 1929; Rosario 1984). A plant in the genus was first collected by Paul Hermann of the Dutch East India Company in Ceylon between 1672 and 1677. He was responsible for giving the vernacular name "Mussaenda" which was later adopted by Linnaeus. In the Philippines, species are found growing at sea level or at 2200 m elevation, growing on dry slopes or in humid, dense forests (Rosario 1984).

Mussaenda cultivars are normally grown as landscape shrubs in tropical and subtropical countries such as the Philippines, India, Taiwan, Australia, and United States (Florida, Hawaii, Puerto Rico, Virgin Islands; Rosario 1984). In southern Florida, the demand for large *Mussaenda* container plants exceeds the supply. In addition, there is some interest to develop *Mussaenda* as a small flowering potted plant in the United States (Bridgen and Cramer 1993). Several *Mussaenda* species are utilized for the medicinal properties of their leaves, however, the majority of them are not utilized commercially.

1.2 Conventional Propagation and Need for Micropagation

The most common method of *Mussaenda* propagation is the rooting of excised softwood terminal cuttings from April until September (Rosario 1984). Cuttings are often treated with a rooting hormone to improve rooting (Cramer 1994). Semi-hardwood and hardwood cuttings can be rooted but with more difficulty. Other asexual propagation such as air layering, ground layering, and arching are used with *Mussaenda* but with limited success. Sexual propagation

of *Mussaenda* cultivars is not attempted because reproductive barriers make seed production extremely difficult (Rosario 1987).

The lack of a dependable propagation method is a setback to the development of *Mussaenda* in the United States (Cramer 1994). The rooting success of a terminal shoot cutting varies greatly depending on the environmental conditions and vigor of the cutting. In addition, cuttings are difficult to root from November until February. Sexual propagation, air layering, ground layering, or inarching are not economical on a commercial basis. Conversely, the microppropagation of *Mussaenda* cultivars produces uniform rooted cuttings in an economical manner.

2 In Vitro Studies and Microppropagation

2.1 Callus Culture

We have attempted to culture axillary buds of *Mussaenda* Queen Sirikit with no success, however, the proliferation of callus from midrib segments of several cultivars has been examined in several studies. Rao et al. (1983) reported that increasing concentrations of indole-3-acetic acid (IAA) decreased callus growth of *Mussaenda rosea* L. cell cultures. In 1989, Panda et al. observed the best callus response from *Mussaenda* Queen Sirikit and Rosea midrib segments cultured on Murashige and Skoog (MS) basal medium (1962) supplemented with $2.22\mu\text{M}$ 6-benzylaminopurine (BAP). Later, Cramer (1994) determined that IAA was required in the culture medium in order to generate callus from Doña Hilaria or Doña Evangelina midribs. In addition, fewer Doña Hilaria or Doña Evangelina midrib segments developed callus than Queen Sirikit or Doña Luz segments when midribs were cultured on a medium without IAA. Furthermore, the percentage of midribs forming callus increased for all four cultivars as the IAA concentration in the medium increased from 0 to $20\mu\text{M}$. In the same study, midrib sections of all four cultivars developed callus equally well on media in which the BAP concentration in the medium ranged from 0 to $50\mu\text{M}$ (Cramer 1994).

2.2 Somatic Embryogenesis

In 1993, Das et al. generated somatic embryos from Queen Sirikit and Rosea callus cultures initiated on MS medium supplemented with $13.3\mu\text{M}$ BAP and $11.4\mu\text{M}$ IAA for 4 weeks, then subcultured to MS medium supplemented with $8.9\mu\text{M}$ BAP, $0.57\mu\text{M}$ IAA, and $56.8\mu\text{M}$ ascorbic acid for an additional 4 weeks. Somatic embryos were produced on 50% of the callus cultures with this protocol. In 1994, Cramer produced somatic embryos after 8 weeks from

Queen Sirikit midrib segments cultured on MS medium supplemented with 87.7 mM sucrose, 5 g l⁻¹ agar, and various concentrations of IAA and BAP (Fig. 2). He observed a decrease in the average number of Queen Sirikit somatic embryos produced per tube as the IAA concentration in the medium increased from 0 to 20 μ M. In addition, BAP concentration between 5 and 10 μ M produced the greatest number of Queen Sirikit somatic embryos per tube. Above 10 μ M, the number of somatic embryos produced per tube decreased as the BAP concentration increased. Cramer (1994) also obtained somatic embryos from Doña Aurora callus cultures by culturing midrib segments for 8 weeks on a medium containing one half inorganic MS salts, 1× MS vitamins, 87.7 mM sucrose, 7 g l⁻¹ agar and 5 μ M BAP (Fig. 3). In addition, Doña Luz callus cultures produced somatic embryos after 12 weeks when cultured on a medium containing MS salts and vitamins, 87.7 mM sucrose, 7 g l⁻¹ agar, 5 μ M BAP and 5 μ M IAA for 8 weeks, then subcultured to a medium without plant growth regulators (Cramer, 1984). However, somatic embryos were not formed from callus cultures of Doña Hilaria and Doña Evangelina. (Cramer and Bridgen 1994).

Das et al. (1993) germinated Queen Sirikit and Rosea somatic embryos on one-half strength MS medium supplemented with 37.03 μ M adenine sulfate and 2% (w/v) sucrose. Cramer (1994) germinated Queen Sirikit somatic em-

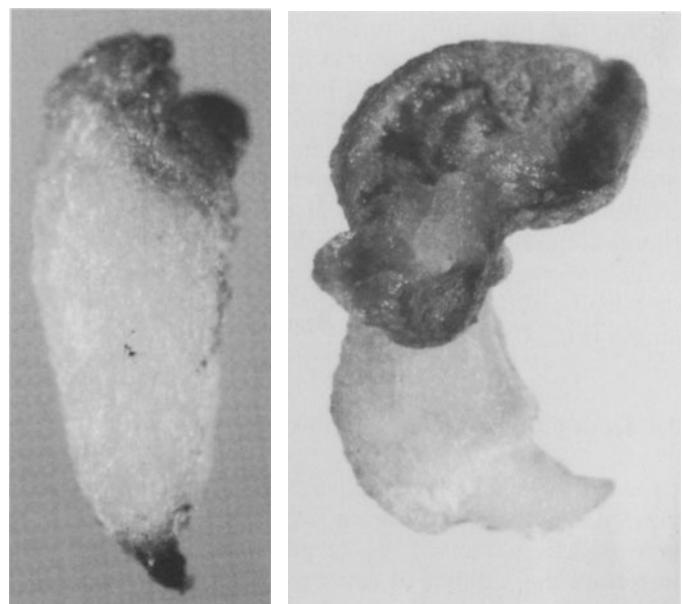


Fig. 2. Torpedo somatic embryo of *Mussaenda* Queen Sirikit

Fig. 3. Germinated *Mussaenda* Doña Aurora somatic embryo removed from callus after 8 weeks of culture on medium containing one-half inorganic MS salts, 1× MS vitamins, 87.7 mM sucrose, 7 g l⁻¹ agar and 5 μ M BAP. (Cramer 1994)

bryos on MS basal medium supplemented with 87.7 mM sucrose and 5 g agar l⁻¹ at pH 5.6.

2.3 Plant Regeneration and Multiplication

Two studies have examined shoot regeneration from callus cultures and shoot multiplication from in vitro shoot tip cultures. Panda et al. (1989) observed the greatest number of Queen Sirikit and Rosea shoots regenerated from callus after 60–70 days in culture grown on MS medium supplemented with 13.32 μ M BAP, 11.42 μ M IAA and 56.8 μ M ascorbic acid. In addition, they were able to proliferate callus-derived shoots by placing nodal cuttings of these shoots on MS medium supplemented with 217 μ M adenine sulfate. In a later study, Cramer (1994) cultured somatic embryo-derived Doña Luz shoot tips on a medium comprised of MS basal salts, 0.56 mM myo-inositol, 1.2 μ M thiamine-HCl, 87.7 mM sucrose, 7 g agar l⁻¹, one of six levels of BAP (0, 2.5, 5, 10, 20, 40 μ M), one of two levels of NAA (0, 1 μ M), and one of two levels of adenine sulfate (0, 217.0 μ M) (Fig. 4). After 6 weeks, he observed a quadratic increase in the total number of shoots produced per culture as the BAP concentration in the medium increased from 0 to 25 μ M (Fig. 5). However, fewer shoots were regenerated per culture when the BAP concentration exceeded 25 μ M. In addition, the absence of NAA and adenine sulfate from the culture medium resulted in more shoots being produced per culture for all BAP treatments than if one or both compounds were present in the medium (Fig. 3). The production of axillary and adventitious shoots per culture after 6 weeks exhibited a similar response over medium BAP concentrations as the total number of shoots. In addition, Cramer (1994) observed a decrease in the average shoot length per culture as the BAP concentration in the medium increased from 0 to 40 μ M (Fig. 6). However, shoots cultured on a medium with 2.5, 5, 10, 20, or 40 μ M BAP, failed to differ in their average shoot length after 6 weeks. In addition, the presence of NAA or adenine sulfate in the culture medium did not influence average shoot length (Fig. 4).

2.4 Root Formation and Plantlet Acclimation

Cramer (1994) observed a decrease in the number of roots formed from in vitro “Doña Luz” shoot tips as the BAP concentration in the culture medium increased from 0 to 40 μ M. The addition of 1 μ M NAA to the culture medium increased the number of roots produced per shoot tip. In addition, in vitro shoots were successfully rooted in moist, sterile, soilless Metro Mix 510 (Grace Sierra, Milpitas, CA., USA) and high humidity environment (Cramer 1994). Rooted plantlets were acclimatized under high humidity conditions in a growth chamber for 4 weeks before transfer to a propagation greenhouse. Plantlets were maintained in a propagation greenhouse for 3 weeks before gradual exposure to normal greenhouse conditions.

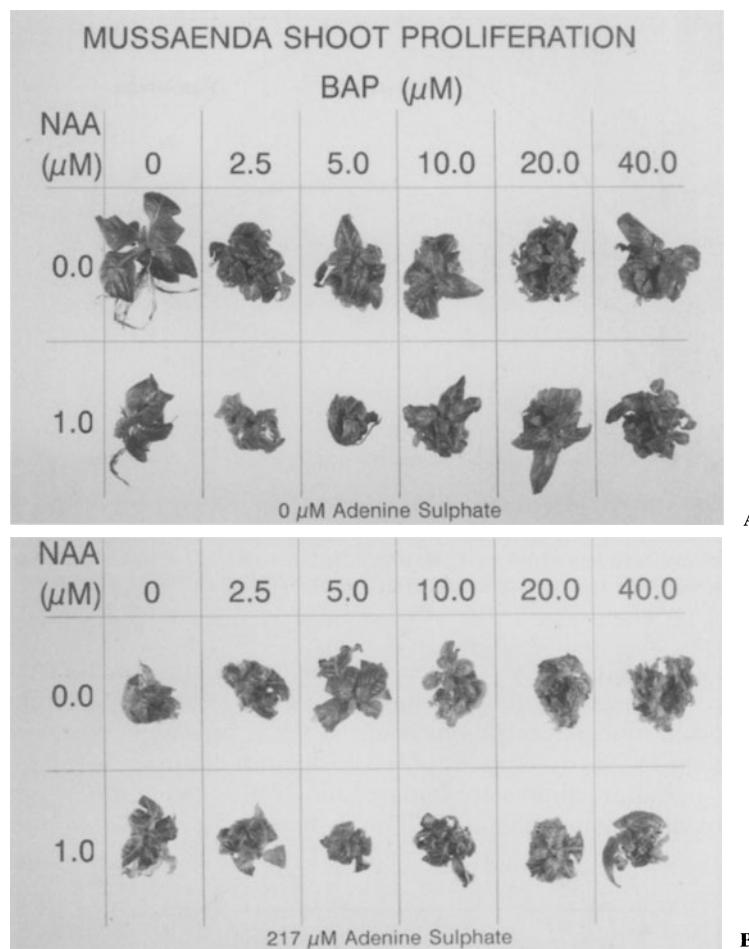


Fig. 4. *Mussaenda* Doña Luz shoot tips after 6 weeks of culture on MS basal medium supplemented with 0 to 40 μ M BAP, plus 0 or 1 μ M NAA, 0 μ M adenine sulfate (A) or 217 μ M adenine sulfate (B) or 1 μ M NAA and 217 μ M adenine sulfate. (Cramer 1994)

2.5 Material and Media

Plant material used as a source for explants should be maintained clean and disease-free. In addition, stock plants should not be stressed from nutritional deficiencies, toxicities, water excesses or deficiencies. In selecting leaves for explants, recently matured, healthy, and turgid leaves are excised from healthy stock plants.

Excised leaves are surface sterilized with 2% commercial bleach solution (5.25% sodium hypochlorite) and 0.2% Tween 20 for 5 min with constant agitation. After one rinse in sterile deionized water, leaf midribs are excised

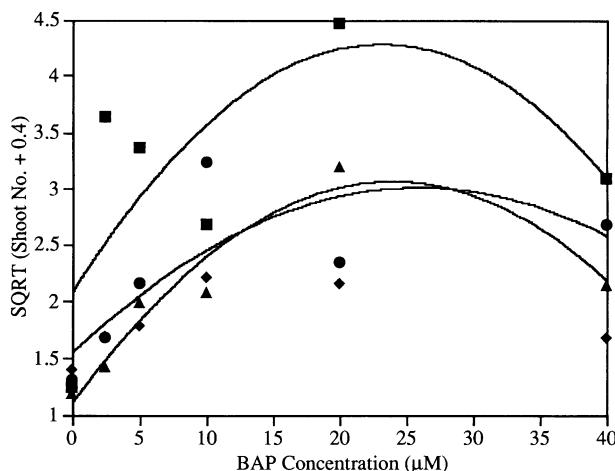


Fig. 5. Observed means (symbols) and polynomial regression curves (lines) for the total number of shoots proliferated by *Mussaenda Doña Luz* shoot tips cultured on MS basal medium supplemented with 0 to 40 μ M BAP (■) plus 1 μ M NAA (▲), 217 μ M adenine sulfate (●) or 1 μ M NAA and 217 μ M adenine sulfate (◆) after 6 weeks. (Cramer 1994)

and placed in 0.1% commercial bleach solution and 0.2% Tween 20 for 15 min with constant agitation. Longer sterilization times and/or higher sodium hypochlorite concentrations result in tissue browning. Afterwards, midribs are rinsed with two changes of sterile deionized water.

Callus cultures are initiated on a callus medium (CM) comprised of MS basal salts and vitamins, 87.7 mM sucrose, 5 g agar l⁻¹, 5–10 μ M BAP, and 5–

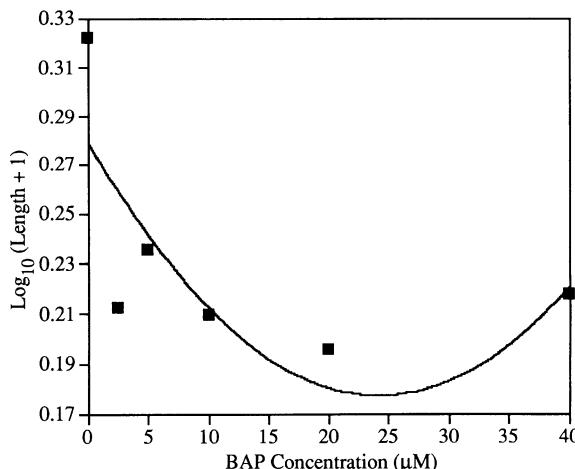


Fig. 6. Observed means and polynomial regression curve for the \log_{10} (average shoot length + 1) of *Mussaenda Doña Luz* shoots cultured on MS basal medium supplemented with 0, 2.5, 5, 10, 20, or 40 μ M BAP for 6 weeks. (Cramer 1994)

10 μ M IAA. The medium is adjusted to pH 5.6 prior to autoclaving. The medium (SEM) used for initiating Queen Sirikit and Doña Luz somatic embryos is identical to (CM) except that IAA is removed. Somatic embryos are germinated on CM without BAP and IAA (GM). The inorganic salts in the CM and SEM are reduced in half in order to produce somatic embryos from Doña Aurora callus cultures. Shoot proliferation medium (SPM) consists of MS basal salts, 0.56 mM myo-inositol, 1.2 μ M thiamine-HCl, 87.7 mM sucrose, 7 g agar l⁻¹, and 10–20 μ M BAP. The medium is adjusted to pH 5.8. All media are autoclaved at 1 kg cm⁻² and 121 °C for 15 mins.

2.6 Establishment of Cultures

Callus cultures are established by placing disinfected 3–5 mm midrib sections onto callus medium (CM). Cultures are grown at a temperature of 25–29 °C with cool white fluorescent lights (210–480 μ mol m⁻² s⁻¹) at a photoperiod of 16–24 h. White, light green, and green calli develop after 2 weeks at the cut ends of midribs (Fig. 7). Callus cultures are subcultured to fresh media every 4–5 weeks to prevent callus browning. In addition, actively growing callus cultures are divided with every subculture. *Mussaenda* cultivars differ in callus growth rates, callus color, and rate of callus browning. Cultures of Queen

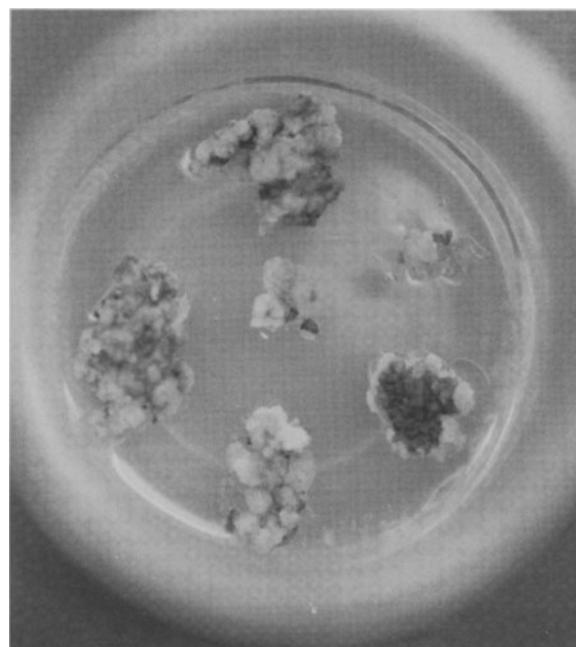


Fig. 7. *Mussaenda* Doña Aurora callus derived from midrib segments cultured on medium containing one-half inorganic MS salts, 1× MS vitamins, 87.7 mM sucrose, 7 g l⁻¹ agar and 5 μ M BAP

Sirikit, Doña Luz and Doña Evangelina grow quicker and produce more friable callus than Daña Hilaria cultures. Doña Evangelina callus cultures turn brown in color sooner than other cultivars.

Somatic embryos appear on callus cultures of Queen Sirikit, Doña Aurora and Doña Luz after 8 weeks of culture. Callus cultures continue to produce somatic embryos for 7 weeks. Normally, somatic embryos germinate on the callus before they are noticed. Germinated and nongerminated somatic embryos are removed from the callus and are cultured on a germination medium (GM) which does not have plant growth regulators. Somatic embryos develop into small plantlets after 4–5 weeks and roots develop at the base of the plantlets during this time.

2.7 Regeneration and Acclimatization of Plants

Individual, aseptic 5-mm shoot tips are excised from somatic embryo-derived plantlets and cultured on shoot proliferation medium (SPM). Cultures are grown at a temperature of 18–22°C with cool white fluorescent lights ($54\mu\text{mol m}^{-2}\text{ s}^{-1}$) at a photoperiod of 16hs. Regenerated shoots are excised from cultures after 6 weeks and rooted in a moist soilless growing media such as Metro Mix 510. In vitro rooting is not necessary as shoots will root directly in soilless growth media.

Excised in vitro shoots are placed in a moist soilless growing medium for rooting (Fig. 7) and the humidity around the shoots is maintained above 95%. The light and temperature conditions are maintained constant at the levels used during proliferation. These conditions are accomplished by placing a plastic bag around the shoot container and growing the shoots in a growth chamber. Shoots are watered periodically during this time. Plantlets are removed from the growth chamber after 4 weeks and transported to a propagation greenhouse. Plantlets are misted frequently to maintain a high humidity environment. After 3 weeks, plantlets are slowly acclimatized to normal greenhouse conditions over a span of 7 days. Plantlets are grown using Metro Mix 510 or a 1 Metro Mix 510:1 perlite (by volume) mixture. Plants are gradually transplanted to larger pots as the need arises, and they flower in 25–35 weeks after being removed from in vitro conditions.

3 Summary and Conclusion

The best callus response from *Mussaenda* Queen Sirikit was observed from midrib segments cultured on MS basal medium supplemented with 2.22 μM BAP (Panda et al. 1989). Both Das et al. (1993) and Cramer (1994) generated somatic embryos from callus cultures grown on MS basal medium plus 5–10 μM BAP and 5–10 μM IAA for 4 weeks then transferred to MS medium

supplemented with reduced concentrations of BAP (5 μ M) and IAA (0–1 μ M) for an additional 4 weeks. The greatest number of shoots proliferated from callus was obtained from callus cultures grown on MS medium supplemented with 13.32 μ M BAP, 11.42 μ M IAA and 56.8 μ M ascorbic acid for 60–70 days (Panda et al. 1989). Cramer (1994) observed the greatest proliferation of shoots from in vitro shoot tips cultured on MS basal salts plus vitamins and 10–20 μ M BAP.

Mussaenda is an attractive ornamental shrub with colorful enlarged sepals and has great potential for commercial development. It is normally propagated by terminal cuttings, a slow and unsuccessful propagation technique. The micropropagation of *Mussaenda* cultivars enables the economical and reliable production of uniform propagules all year.

4 Protocol for Microppropagation

Explant. Midrib segments (3–5 mm long).

Medium. Murashige and Skoog basal salts and vitamins (MS) (1962), 5–10 μ M BAP, 5 μ M IAA for 4 weeks, followed by MS, 5–10 μ M BAP for 4 weeks. Somatic embryos placed on MS without plant growth regulators. In vitro shoots placed on MS plus 10–20 μ M BAP for shoot proliferation.

Culture Conditions. Aseptic cultures maintained at 25–29 °C with cool white fluorescent lights (210–480 μ mol m⁻² s⁻¹) at a photoperiod of 16–24 h for callus formation and somatic embryogenesis. Shoot proliferation cultures are grown at 18–22 °C with cool white fluorescent lights (54 μ mol m⁻² s⁻¹) at a photoperiod of 16 h. Shoots are rooted in moist soilless potting mix in the growth chamber with high humidity conditions. Plantlets are slowly acclimatized to normal greenhouse conditions.

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1.17 Micropagation of *Narcissus* (Daffodils)

B.M.R. HARVEY and C. SELBY¹

1 Introduction

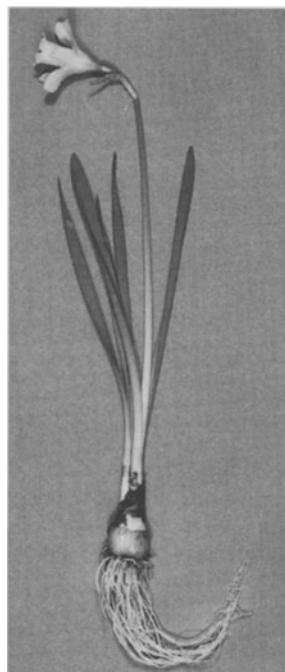
1.1 Botany and Importance

Narcissus species (daffodils, narcissi, jonquils, lent lilies, Chinese sacred lilies) are bulbous monocotyledonous plants of the family Amaryllidaceae, well known for their attractive flowers (Fig. 1). Characteristically, the flower has six outer perianth segments (three sepals and three petals) with a central corona (trumpet or cup) and is born on a leafless scape (stem). The bulb is complex, having both swollen leaf bases and leaf scales surrounding the bud which gives rise to new leaves and the inflorescence. All are borne on a compressed stem (basal plate or disc) which gives rise to adventitious roots (Rees 1972).

The species thought to be most primitive (e.g. *N. elegans* and *N. humilis*) are found in the Iberian peninsula and North Africa (Jefferson-Brown 1991) and the majority of species appear native to countries around the Mediterranean. However, apparently wild populations of *N. pseudonarcissus* occur as far north as Britain and *N. tazetta* extends to India, China and Japan, possibly due to distribution along trading routes. In many areas it is impossible to be certain whether populations are native or naturalised. Spontaneous hybridisation between wild species, between wild species and naturalised "garden escapes", and extensive horticultural breeding make the taxonomy of *Narcissus* very difficult. The Fernandes classification (1968) lists 16 sections of the genus with a total of 62 species and this is the taxonomy and nomenclature employed by the Royal Horticultural Society, UK, in its work on the International Daffodil Register (Kington 1989). However, flora Europea (Webb 1980) recognises only eight sections with a total of 26 species and four inter sectional hybrids.

Despite numerous mentions of *Narcissus* in Herbals from 1530 onwards (listed by Pugsley 1993) and much early work on its taxonomy (also described by Pugsley 1993), *Narcissus* only became an important bulb crop in the late nineteenth century. It is now grown commercially in very many countries throughout the world, the major producers of outdoor crops being the United

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Fig. 1. Flowering plant of *Narcissus pseudonarcissus* cv. Little Gem, a miniature in division 1 of the horticultural classification (see Table 1) of *Narcissus*. (Harvey, unpubl.)

Fig. 2. "Mini chip" explant of cv. Hawera (division 5) showing the initiation of new leaves from the basal plate. (Harvey, unpubl.)

Kingdom and The Netherlands. It is not only important as a garden perennial, used for formal planting and for naturalisation in parks and public places, but also as a cut flower and as an indoor pot plant, bulbs being pre-treated ("forced") to ensure early flowering. Interest in *Narcissus* is so great that as many as 23 000 cultivars are listed in the International Daffodil Checklist (Kington 1989) and more than 150 new cultivars are added to the International Daffodil Register every year. For horticultural purposes *Narcissus* cultivars are classified into twelve divisions, as shown in Table 1.

In addition to its horticultural importance the genus *Narcissus* provides many compounds with cosmetic and medicinal applications (Hanks 1993). Infusions of *Narcissus* flowers are used traditionally in Spain for treatment of coughs and colds as well as for their emetic properties (Bastida et al. 1994). The traditional medicinal use of *Narcissus* has led to an extensive study of the alkaloids of species native to Catalonia, Spain. Numerous alkaloids have been characterised (e.g. Viladomat et al. 1992; Bastida et al. 1993; Codina et al. 1993), and many of these are considered to have valuable biological activity such as anti-mitotic, anti-tumour or anti-leukaemic properties. In addition, galanthamine has been found at considerable concentrations in some *Narcis-*

Table 1. The horticultural classification of *Narcissus*. (After Kington 1989 and Royal Horticultural Society 1993)

Division	Description
1	<i>Trumpet daffodils of garden origin</i> One flower to a stem, corona as long as or longer than the perianth segments
2	<i>Large-cupped daffodils of garden origin</i> One flower to a stem; corona more than one-third, but less than equal to the length of the perianth segments
3	<i>Small-cupped daffodils of garden origin</i> One flower to a stem; corona not more than one-third the length of the perianth segments
4	<i>Double daffodils of garden origin</i> One or more flowers to a stem, with doubling of the perianth segments or corona or both
5	<i>Triandrus daffodils of garden origin</i> Characteristics of <i>N. triandrus</i> clearly evident: usually two or more pendent flowers to a stem; perianth segments reflexed
6	<i>Cyclamineus daffodils of garden origin</i> Characteristics of <i>N. cyclamineus</i> clearly evident: one flower to a stem; perianth segments significantly reflexed; flower at an acute angle to the stem, with a very short pedicel
7	<i>Jonquilla daffodils of garden origin</i> Characteristics of the <i>N. jonquilla</i> group clearly evident: usually one to three flowers to a rounded stem; leaves narrow, dark green; perianth segments spreading not reflexed; flowers fragrant
8	<i>Tazetta daffodils of garden origin</i> Characteristics of the <i>N. tazetta</i> group clearly evident: usually 3 to 20 flowers to a stout stem; leaves broad; perianth segments spreading not reflexed; flowers fragrant
9	<i>Poeticus daffodils of garden origin</i> Characteristics of the <i>N. poeticus</i> group without admixture of any other: usually one flower to a stem; perianth segments pure white; corona usually disc-shaped, with a green rim or yellow centre and a red rim; flowers fragrant
10	<i>Species, wild variants and wild hybrids</i> All species are wild or reportedly wild variants and hybrids, including those with double flowers
11	<i>Split-corona daffodils of garden origin</i> Corona split, usually for more than half its length <ul style="list-style-type: none"> a) Collar daffodils – corona segments opposite the perianth segments and usually in two whorls of three b) Papillon daffodils – corona segments alternate to the perianth segments and usually in a single whorl of six
12	<i>Miscellaneous daffodils</i> All daffodils not falling into one of the foregoing divisions

sus species (Bastida et al. 1987). This alkaloid is used to enhance athletic performance (Bastida et al. 1990) and is proposed as a treatment for Alzheimer's disease. Its efficacy will be assessed in a clinical trial starting in summer 1995 and involving five European countries (Bonner 1995).

1.2 Conventional Propagation and Need for Micropropagation

Many wild populations of *Narcissus* produce seed prolifically, but hybrids (often polyploid) do not breed true and bulbs must be propagated vegetatively. However, *Narcissus* bulbs are long lived and multiply very slowly. As the outer parts of the bulb senesce, new bulb units are initiated internally. These are of two types, terminal and lateral. Each terminal unit will contain a terminal and a lateral unit the following year, but each lateral unit will contain only a terminal unit in the following year (Rees 1969). This results in bulb multiplication according to a Fibonacci series, i.e. 1, 2, 3, 5, 8 etc. In practice, rates of natural vegetative propagation of *Narcissus* bulbs are only about 1.6-fold per year (Rees 1992) and the percentage weight increases of field crops differ very greatly between cultivars, even for cultivars within the same Divisions of the horticultural classification (ADAS 1982).

Twin scaling of *Narcissus* was developed to accelerate vegetative propagation and has been reviewed by Hanks and Rees (1979). In summary, it involves cutting dormant flowering size bulbs into small pieces, each with two adjacent bulb scales attached to the bulb basal plate tissue. Fifty to 100 twin scales can be prepared from each bulb, depending on its size. When incubated in moist substrate (e.g. vermiculite), each twin scale forms a small bulblet which can be transferred to soil and grown on to produce a flowering size bulb. The main problems are that the technique is laborious, the propagules must be carefully protected from disease, and that the small bulblets take 3 to 4 years to reach flowering size.

Chipping is a simpler version of twin scaling (Vreeburgh 1986). In this technique bulbs are cut into 4, 8 or 16 segments depending on bulb size and then may be incubated in vermiculite or planted directly into soil. The segments produce larger bulblets than those produced by twin scaling and may reach flowering size more quickly. Hanks (1993) states that realistic multiplication rates for both methods are three-to four-fold per annum, taking into account the losses due to disease etc. and the time required for bulblets to reach flowering size.

Although the methods produce similar multiplication rates, chipping is in more general commercial use (Rees 1992). This is because mechanised cutters allow a throughput of several hundred bulbs per hour and the bulblets produced from chips are more robust than those produced from small twin scales. Direct planting into the field may also reduce costs.

Twin scaling and chipping are considerable improvements on natural rates of propagation of *Narcissus* bulbs, enabling an increase from 1 to 1000 in 5–6.5 years (Hanks 1993) instead of 15–25 years (Vreeburgh 1986). However, even better multiplication rates may be achieved by micropropagation and this is desirable for virus-tested bulbs and for new cultivars with improved flower characteristics (e.g. colour, fragrance, longevity), length and sturdiness of stems, or other valuable attributes. In vitro culture and micropropagation could also have an important role in production of pest- and disease-resistant cultivars by use of modern molecular techniques, and possibly in multiplica-

tion of genotypes which produce high concentrations of valuable secondary metabolites.

2 Micropagation

There seem to be no publications on in vitro culture of *Narcissus* prior to the 1970s. Use of meristem tip culture for virus elimination from *N. tazetta* cv. Grand Soleil d'Or was attempted as early as 1963 (Stone 1973), but survival of the explants was very poor and after transplantation to soil only ten bulbs were produced from a total of 345 excised meristems. These bulbs took 5 years to reach flowering size and were subsequently multiplied by chipping. The resultant virus free stock showed marked improvements in vigour, flower quality and yield. Since then, meristem tip culture has been used in many *Narcissus* virus elimination programmes (Hanks 1993).

Direct organogenesis from bulb explants was reported by Hussey (1975, 1976, 1977), and by Hussey and Wyville (1973) for various species and cultivars of the Liliaceae, Iridaceae and Amaryllidaceae. However, Hussey found that high levels of cytokinin ($2\text{--}32\text{ mg l}^{-1}$ BA) were needed to overcome apical dominance in *Narcissus* cultures and rooting was inhibited by the cytokinin concentrations required to promote branching (Hussey 1976). Seabrook et al. (1976) were the first to publish a detailed study of initiation and multiplication of shoot clump cultures produced directly from bulb explants and from ovary callus of *Narcissus* cultivars. They also mentioned that strong apical dominance was a problem in shoot clump cultures, restricting multiplication, and that *Narcissus* plantlets produced in vitro tended to be difficult to root and grow in soil.

Hussey (1977) queried whether the shoots arising from bulb explants were axillary or adventitious and stated that continued rapid shoot production occurred only when a "very callus-like reaction" occurred. Subsequently (1982), he published a detailed investigation of the in vitro culture of *Narcissus* and demonstrated by microscopy that new shoots arose directly from bulb explant tissue. Genetic instability therefore seemed unlikely to be a problem. Hussey (1982) also reported a method for in vitro production of *Narcissus* bulblets which survived transplantation to soil. The development of a complete micropagation system was therefore possible and, although genotypes differed greatly in responsiveness (Seabrook et al. 1976; Hussey 1982), the methods were used for six cultivars of division 2, and one of division 3.

Other than the use of meristem tip cultures for virus elimination, most of the more recent in vitro studies of *Narcissus* are based on the methods developed by Seabrook et al. (1976) and Hussey (1982). Publications on in vitro culture and micropagation of *Narcissus* are summarised in Table 2, and discussed below.

Table 2. Summary of the in vitro studies/micropagation of *Narcissus* species and cultivars (divisions shown in parentheses)

Reference	<i>Narcissus</i> species/cultivar (division)	Explant used		Tissue	Observations
		Origin			
Stone (1973)	<i>N. tazetta</i> cv. Grand Soleil d'Or (8)	Dormant bulb	Shoot meristem tips	Twin scale	Low success rate, 22 plants from 144 meristems
Hussey (1975)	<i>N. pseudonarcissus</i> , hybrid cvs. Actaea (9) Cheerfulness (4), Cragford (8), Edward Buxton (3), Golden Harvest (1), Scarlet Elegance (2)	Dormant bulb Sprouting bulb	Leaf Stem Ovary wall		Adventitious buds form freely No reaction No reaction Callus formation
Hussey (1976)	Cvs. Dutch Master (1), King Alfred (1), Rembrant (1)		In vitro plantlets		Branching promoted by $\geq 2 \text{ mg l}^{-1}$ BA
Seabrook et al. (1976)	Cvs. Chinese White (3), Forerunner (1), Fortune (2), Grand Soleil d'Or (8), King Alfred (1), Lord Nelson (1), Mount Hood (1)	Dormant bulbs, cold-treated bulbs	All parts		Best organogenesis and growth with leaf base explants from cold treated bulbs. Cytokinin and auxin levels and ratios important. Cultivar differences in responses. Apical dominance problems
Hussey (1977)	Cvs. Barrett Browning (3), Brabazon (1), Carlton (2), Fortune (2), Golden Harvest (1), Pink Smiles (2), Scarlett O'Hara (2), Sealing Wax (2), Sempre Avanti (2)	Dormant bulb Just sprouted bulb	Bulb scale with basal plate Scape sections inverted		Bulbil and plantlet production favoured by BA + NAA (both explant types) Cultivar differences in responses. Apical dominance problems
Hosoki and Asahira (1980)	<i>N. tazetta</i> cv. Geranium (8), hybrid cv. Fortune (2)	Sprouting bulb	Scape Ovary Leaf Basal disc		Best organogenesis from scape. NAA + BA suppressed callus and promoted bud formation. Geranium responded much better than Fortune

Hussey (1982)	Cvs. Barrett Browning (3), Carlton (2), Fortune (2), Golden Harvest (1), Hollywood (2), Pink Smiles (2), Scarlett O'Hara (2), Sempre Avanti (2)	Cold-treated sprouting bulbs	Bulb scale with basal plate	Shoot formation best with NAA + BA. Cultivar differences in responses. Apical dominance problems. Sprouting bulbils required for successful planting out
Seabrook and Cummings (1982)	Cv. Lord Nelson (1)	In vitro shoot clump cultures	Split clumps	Shoot proliferation best at constant 25°C
Steinitz and Yahel (1982)	<i>N. tazetta</i> cv. Grand Soleil d'Or (8)	Bulbs In vitro bulbils	Twin scales Split bulbils	Direct bulbil formation on explant, stimulated by activated charcoal, inhibited by BA + NAA.
Squires and Langton (1990)	Cvs. Armyne (2), Bridal Crown (4), Carlton (2), Dutch Master (1), St. Keverne (2), Tête-a-Tête (6), Yellow Sun (2)	Dormant bulbs	Inner bulb unit segments	Sprouted, rooted bulbils establish successfully in field if >0.25 g
Seabrook (1990)	—	—	—	Complete microppropagation system; initiation, multiplication, bulbil production by senescing cultures, cold treatment to break dormancy before transplanting
Chow (1990)	23 cvs. from divisions 1-5, 7	Dormant bulb Sprouting bulb	Minichips Inner bulb unit segments	Review
Squires et al. (1991)	Cvs. Armyne (2), Carlton (2), Dutch Master (1), St. Keverne (2), Tête-a-Tête (6), Yellow Cheerfulness (4), Yellow Sun (2)	As Squires and Langton (1990)	Complete microppropagation system; initiation, multiplication, rapid production of non-dormant bulbils. Differences in cultivar responses	Semi-commercial trial, best transplantation success with bulbils >0.2g

Table 2. Continued

Reference	Narcissus species/cultivar (division)	Explant used		Observations
		Origin	Tissue	
Bergoñón et al. (1992)	<i>N. papyraceus</i>	Pre-flowering bulbs	Axillary shoots	Complete micropropagation systems; initiation, multiplication by liquid shake culture, transplantation with or without rooting phase on agar solidified medium
Chow et al. (1992b)	Cvs. Carlton (2), Ice Follies (2), Liberty Bells (5), St. Keverne (2), Thalia (5), Tresamble (5), Yellow Sun (2)	In vitro shoot clumps	Split clumps	Shoot multiplication improved by simple cutting treatment which breaks apical dominance
Hol and van der Linde (1992)	Cvs. Carlton (2), Dick Wilden (4), Golden Harvest (1), St. Patrick's Day (2), Tahiti (4), Tête-à-Tête (6)	Dormant bulbs \pm 4 week storage at 30°C and 1 h HWT at 54°C	Large twin scales, inverted	Hot-water treatment at 54°C reduced contamination but not regeneration
Staikidou (1992)	Cvs. Carlton (2), Hawera (5), St. Keverne (3) Yellow Sun (2)	In vitro shoot clumps	Split clumps Single leaf units with basal tissue	Study of factors affecting bulbil development on agar solidified medium
Chow et al. (1993)	Cvs. Hawera (5), St. Keverne (3)	In vitro shoot clumps	Split clumps Single leaf units with basal tissue	Organogenesis from basal plate tissue is similar in shoot clumps and normal bulbs.
Staikidou et al. (1994)	Cvs. Hawera (5), St. Keverne (3)	In vitro shoot clumps	Split clumps Single leaf units with basal tissue	Single leaves excised with basal tissue are highly organogenic Single leaf units are more sensitive to sucrose and hormones than shoot clumps. Cultivars differ in auxin response

2.1 Initiation of Cultures

Many plant parts have been used as explants for the initiation of in vitro cultures, but by far the most widely used and responsive are sections of non-dormant bulbs containing the basal sections of leaves or scales (5–15 mm in height) with a segment of base plate tissue left attached (Fig. 2). Terms such as “twin scales” or “mini-chips” have been used to describe these explants and they have been prepared from either most of the basal tissues of the bulb (Hol and van der Linde 1992) or from as little as the inner terminal bulb units (Squires and Langton 1990). Relatively large numbers of explants (50–60) can be prepared from a single bulb when most of the bulb’s basal zone is used and small twin scale explants prepared (Steinitz and Yahel 1982). Explants from cold-treated non-dormant bulbs showed better growth and survival (Seabrook et al. 1976) and produced more shoots (Hussey 1982) than dormant bulb tissues.

Initially, explant preparation usually involves removal of the roots and outer bulb layers and washing the bulbs with detergent and water. Bulbs cleaned up in this way are then either surface sterilised whole (Hussey 1982), or after careful dissection of the bulb units (Squires and Langton 1990), or after the complete removal of the apical part of the bulb (Steinitz and Yahel 1982; Hol and van der Linde 1992). Surface sterilisation procedures using a combination of careful dissection of the outer bulb layers and ethanol wiping (Hussey 1982), or various hypochlorite treatments either alone (Squires and Langton 1990) or in combination with short ethanol dips (Seabrook et al. 1976) have all been used successfully to introduce tissues into culture. Rates of contamination using such methods are not always reported, although Squires and Langton (1986/87, 1990) quote infection rates of 20%, a value confirmed by Chow (1990) using their methods.

Microbial contamination can often be a problem with plant tissues and organs, such as bulbs, formed underground (Hol and van der Linde 1992 and references therein), and to some extent this can be combated by using severe surface sterilisation treatments (Seabrook et al. 1976) or culture media containing fungicides (Cohen and Whitehead 1987). Hol and van der Linde (1992) suggest that the ineffectiveness of these approaches indicate that fungi may be located within the plant tissues. Such internal fungal contamination was demonstrated when surface sterilised explants prepared from the inner third of *Narcissus* bulbs (cv. Ice Follies) were macerated and plated onto malt agar. Saprophytic fungi identified in the macerates included *Penicillium*, *Rhizopus*, *Oidium*, *Geotrichum* and *Botryosporum*. Unmacerated explants appeared sterile when incubated on the same medium (McCracken, unpubl.).

Hol and van der Linde (1992) developed a hot-water treatment, applied by immersing bulbs in water at 54 °C for 1 h, which greatly reduced fungal contamination in cultures initiated from *Narcissus* bulb tissues. When hot-water treatment was used prior to conventional surface sterilisation and dissection of explants it decreased infection rates from 45 to 3% with the cultivar Golden Harvest and by similar amounts with five other cultivars (Hol and van der Linde 1992). The effectiveness of hot-water treatment was also demon-

strated by Chow (1990); using this technique six *Narcissus* cultivars showed acceptably low infection rates (Table 3).

Use of lower water temperatures (50°C) failed to control the contamination, whilst higher temperatures (58°C) prevented shoot formation with the cv. Golden Harvest (Hol and van der Linde 1992). It is essential to harden bulbs to higher temperatures before subjecting them to hot-water treatment and this is usually achieved by pre-conditioning them at 30°C for a period of at least 4 weeks. Without temperature pre-conditioning hot-water treatment severely damages bulb tissues and reduces their responsiveness in culture. Similarly, sprouting bulbs should not be subjected to hot-water treatment because of the risk of damaging the tissues. Hol and van der Linde (1992) reported that if these precautions are taken hot-water treatment has little effect on the viability and shoot-producing abilities of explants. However, Chow (1990) reported that induction regimes including hot-water treatment in the sterilisation procedure reduced the number of shoots formed (see Table 3) and that explants had an increased tendency to form bulbils rather than shoots during culture initiation.

Bulb explants other than "twin scales" and "mini-chips" have been used much less frequently, but sections of scape and ovary in particular have been reported to be organogenic (Hosoki and Asahira 1980; Seabrook and Cumming 1982; Seabrook 1990). Basal plate tissue cultured alone and isolated scale pieces were much less regenerative (Hussey 1975; Hosoki and Asahira 1980). There are some suggestions in the literature that inverting tissue on the culture medium can be beneficial. For instance, Seabrook (1990) stated that it

Table 3. Initiation of in vitro cultures of *Narcissus* using the methods described by Squires and Langton 1990 (method I) and by Hol and van der Linde 1992 (method II): infection and organogenesis after two 5-week culture passages. (After Chow 1990)

Cultivar	Initiation method I ^a			Initiation method II ^b		
	Infection (%)	Numbers initiated		Infection (%)	Numbers initiated	
		Shoots	Bulbils		Shoots	Bulbils
Carlton	18.8	1.9	0.1	3.1	0.3	1.1
Fortune	34.4	3.3	0.1	12.5	0.3	1.1
Ice Follies	21.9	3.3	0	0	1.9	0.8
St. Keverne	12.5	6.7	0.1	0	1.0	1.1
Yellow Sun	21.9	2.8	0.2	3.1	0.6	1.0
Mean	21.9	3.6	0.1	3.7	0.8	1.0

^a Bulbs were stored at 17°C for 4 weeks, before dissecting out and surface-sterilizing the inner bulb unit. Each unit was cut into eight "minichips" which were inoculated upright and cultured at 20°C with a 16-h photoperiod. The initiation medium was MS salts and vitamins with 30 g l⁻¹ sucrose, 0.54 µM NAA and 4.44 µM BA.

^b Bulbs were preconditioned at 30°C for 4 weeks, hot-water treated at 54°C for 1 h, trimmed and halved longitudinally before surface sterilization and then cut into eight "minichips" which were inoculated inverted, and cultured at 20°C in darkness. The initiation medium was the same as for method I.

was essential to invert scape sections, whilst Hosoki and Asahira (1980) induced the formation of large numbers of shoots on such explants inoculated basally into the medium. Twin scale explants are usually inoculated with the basal tissues in the medium except for Hol and van der Linde (1992) who used inverted explants. Recent experiments (Harvey, unpubl.) indicate that inversion of mini-chip explants at inoculation does not improve initiation of shoot clump cultures of division 1 and 2 cultivars and may result in low rates of shoot initiation in the early multiplication passages.

The basal nutrients of Murashige and Skoog (1962; MS) have been used successfully to culture tissues of a wide range of *Narcissus* cultivars with or without minor changes to the organic components (Steinitz and Yahel 1982; Chow 1990; Hol and van der Linde 1992), although earlier studies (Seabrook et al. 1976; Hussey 1982) favoured use of the modifications to the MS medium devised by Ziv et al. (1970) for *Gladiolus* culture. MS macronutrients in combination with the micronutrients and organic additives of Ringe and Nitsch (1968) were used to support adventitious bud formation on a wide range of *Narcissus* explants by Hosoki and Asahira (1980). Seabrook et al. (1976) reported that other basal formulations (Gamborg et al. 1968; Schenk and Hildebrandt 1972) were unsuitable.

Initiation of shoots on bulb explants is usually achieved by a combination of naphthalene acetic acid (NAA) and 6-benzyladenine (BA) although the concentrations and ratios used have been very variable. Seabrook et al. (1976) reported that relatively high concentrations of both regulators ($5.3\mu\text{M}$ NAA and $44\mu\text{M}$ BA) were optimal for shoot initiation on leaf base explants, and that changing the ratio resulted in the formation of either callus or roots. In contrast, Hussey (1982) found that such high NAA and BA concentrations were not essential for shoot initiation and that initiation could be achieved over a wide range of plant growth regulator concentrations and ratios. Similarly, Chow (1990), and Hol and van der Linde (1992) induced proliferating shoot cultures using lower growth regulator concentrations ($0.45\mu\text{M}$ NAA and $4.44\mu\text{M}$ BA) with numerous *Narcissus* cultivars from divisions 1–7.

Steinitz and Yahel (1982) induced direct bulblet development, without an intervening leaf or callus phase, on twin scale explants of *N. tazetta* by culturing in the dark on MS medium free of plant growth regulators. In this system addition of 5 g l^{-1} activated charcoal (AC) increased numbers of bulblets, resulted in the formation of larger bulblets and reduced browning in the cultures. The addition of NAA ($5.4\mu\text{M}$) and BA ($44.4\mu\text{M}$) had no effect in the presence of AC but was completely inhibitory to the formation of bulblets in the absence of AC. Such high concentrations of BA in particular would be expected to repress the initiation of bulblets (see Sect. 2.3).

2.2 Multiplication

Complex shoot clumps (Fig. 3) eventually develop when freshly initiated leaves together with segments of tissues derived from the bulb base plate are

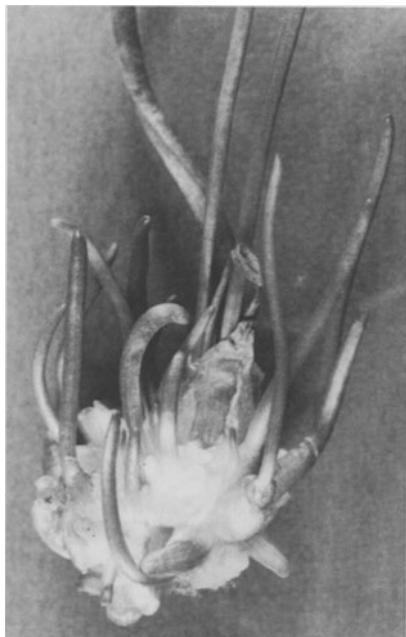


Fig. 3. Shoot clump culture of cv. Hawera (division 5) with leaves at different stages of development growing from white achenophyllous tissues. Actual clump diameter 15 mm. (Harvey, unpubl.)

transferred onto culture media similar to those used for initiation. These clumps are usually composed of a mixture of individual leaves, shoots and small bulbils held together at their bases by a swollen mass of achenophyllous tissue. New leaf primordia arise in meristematic zones at the surface of the achenophyllous tissue close to the bases of existing leaves (Chow et al. 1993). Since no intervening callus phase occurs the risk of somaclonal variation should be low. At high growth regulator concentrations, however, organogenic callus-like tissues can be induced which continue to regenerate leaves over long periods. Meristems on the abaxial surface of leaf bases may also give rise to new leaves (Hussey 1982), although in a later study these abaxial meristems remained in a resting state and did not develop into leaves (Chow et al. 1993).

Multiplication is achieved by the regular subdivision and culturing of shoot clumps, eventually yielding large numbers of shoots per initiation. Assessment of multiplication rates in such complicated shoot clump structures is not a simple task because of difficulties in standardising the size and composition of tissues transferred and of characterising the tissues formed. Squires and Langton (1990) took a simple approach and used the numbers of individual cultures inoculated in each culture passage, averaged over seven to eight transfers, as a measure of multiplication. Counting leaves at the beginning and

end of culture passages has also been used to assess multiplication (Chow 1990; Chow et al. 1992b).

Multiplication rates reported by Squires and Langton (1990) differed between cultivars and ranged from only $\times 1.45$ for cv. Carlton, up to $\times 2.25$ for cv. St. Keverne (both division 2). Chow (1990) also reported that cultivars differed significantly in their multiplication within each culture passage but that the rates fluctuated and cultivars changed their rankings over the first eight subculture passages after initiation. Multiplication rates meaned over several passages were in the same order (ranging from $\times 1.39$ for cv. Tresemble to $\times 2.48$ for Hawera, both division 5) as those found by Squires and Langton (1990).

Higher multiplication rates have been achieved using a single-leaf culture technique (Chow et al. 1993). This involves excision of individual leaves, each with a wedge of basal tissue, from shoot clump cultures. More than five-fold multiplication was obtained in 10 weeks when trimmed single leaf units were cultured in liquid medium (Table 4), but hyperhydration was observed and caused problems when the cultures were planted out. Multiplication of the single leaf cultures on agar-solidified medium was approximately three-fold in 10 weeks (Table 4) and no hyperhydration problems occurred. Thus the single-leaf culture technique could be used to produce numerous small shoot clusters from a single large shoot clump in only 10 weeks. This could have a role in rapid multiplication of scarce material but preparation of the single leaf units is too difficult and laborious for the technique to find a routine use in *Narcissus* micropagation. Shoot clump culture remains the most convenient method of multiplying *Narcissus* in vitro.

Table 4. The cumulative multiplication factors for the first two in vitro passages (0–10 weeks) of single-leaf cultures of two cultivars of *Narcissus*. Three types of explant were either orientated upright or inverted on agar medium or inoculated in liquid culture medium (MS with 30 g l^{-1} sucrose, $0.54\text{ }\mu\text{M}$ NAA and $4.44\text{ }\mu\text{M}$ BA). Intact leaf and leaf base explants included basal achlorophyllous tissue. (After Chow et al. 1993)

Inoculation treatment	Explant type			
	Intact leaf	Leaf base	Leaf lamina	Mean
Upright	3.23	2.31	0	1.85
Inverted	0.11	0.94	0	0.35
Liquid medium	3.00	5.37	0	2.79
Mean	2.11	2.87	0	
Cultivar means	Hawera	1.20	St. Keverne	2.12

Significance of effects and interactions: explant type, $P < 0.001$; inoculation treatment, $P < 0.001$; cultivar, $P < 0.01$; explant type \times inoculation treatment, $P < 0.001$; all other interactions not significant.

Strong apical dominance is an important factor in the suppression of new leaf production in the basal tissues of shoot clump cultures and therefore in determining multiplication rates. Methods of releasing this dominance have included cytokinin treatment (Hussey 1977), splitting or removal of large shoots (Seabrook et al. 1976; Hussey 1982) or trimming all shoots down to the level of the base plate (Chow et al. 1992b). When the last treatment was imposed for four successive subculture passages the shoot clump cultures lost vigour. Therefore alternate hard trimming and recovery passages (no leaf trimming) were necessary to maintain a high multiplication rate, preventing a few large shoots becoming dominant, whilst still allowing the newly formed initials time to recover and grow. The benefits of this regime are shown in Fig. 4.

In some circumstances repeated subculture of shoot clumps will result in senescence and the formation of dormant bulbs (Hussey 1982; Seabrook 1990 and references therein). The time taken to lapse into dormancy can be related to the growth regulator levels used at initiation, shoot clumps initiated with low growth regulator levels becoming dormant after 20–25 weeks compared to

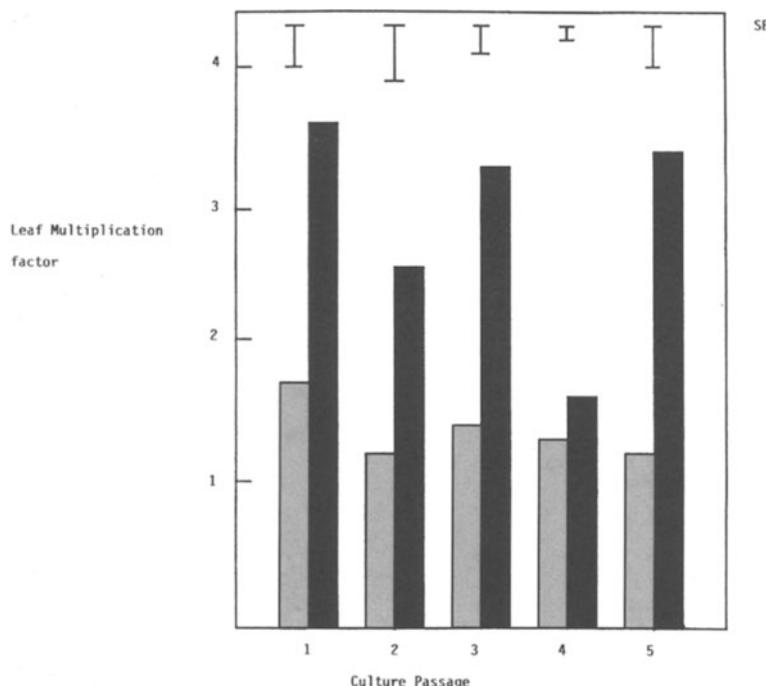


Fig. 4. Effects on leaf multiplication of either normal trimming of large or senescent leaves at the beginning of every culture passage (*shaded columns*) or “severe cutting” down to the basal plate region at alternate culture passages and no cutting during the recovery passages (*solid columns*). “Severe cutting” occurred at the beginning of passages 1, 3 and 5. The results are the mean of four cultivars (Carlton, Ice Follies, St. Keverne and Yellow Sun) and standard errors are shown by bars. (Chow et al. 1992b)

30–35 weeks when higher growth regulator levels were used (Hussey 1982). A progressive increase in dormancy of shoot cultures could be caused by the tissues not being transferred regularly enough (Seabrook 1990), or to the handling procedure at subculture. For instance, Hussey (1982) allowed shoots to become 6–8 mm in diameter and form bulbils before these were split longitudinally to break the apical dominance and stimulate the formation of new shoots. Squires and Langton (1990) used cultural methods similar to those of Hussey, but more regular subculturing (4–6 weeks) and earlier bulbil splitting to destroy growing points. This prevented a decline in multiplication rates and the onset of dormancy. Additionally, Squires and Langton (1990) replaced NAA with 5 µM indol-3-yl-butyric acid in the multiplication medium.

Shoot multiplication has often been performed at the relatively high temperature of 25 °C (Hussey 1982; Seabrook and Cumming 1982) with the tissue constantly exposed to growth regulators. These factors may contribute to eventual leaf senescence and dormancy in shoot clump cultures following repeated subculture. An alternative strategy to avoid or delay the onset of dormancy may be to culture the tissues at a lower temperature, as high temperatures cause dormancy in the normal growth cycle of *Narcissus*. Squires and Langton (1990) did not find a decrease in shoot multiplication at 20 °C. Similarly, cultivars Carlton, Hawera, King Alfred, Flore Pleno and St. Keverne showed no statistically significant temperature effects on multiplication when cultured for 5 weeks at 16, 20 and 24 °C (Harvey, unpubl.) although there was a general trend for greater fresh weight accumulation at 24 °C. In the second culture passage the increase in fresh weight appeared best at 20 °C for three of the five cultivars but this was statistically significant only for one cultivar (Table 5). It had been expected that reducing the temperature would stimulate rooting, as in normal development of *Narcissus* (Jennings and de Hertogh 1977), and that this might improve growth and multiplication

Table 5. Total fresh weight of shoot clump cultures of *Narcissus* cultivars as percent of initial fresh weight for the second of two 5-week passages at 16, 20 or 24 °C. Means are shown \pm standard error. There were no statistically significant differences in shoot multiplication, rooting or fresh weight accumulation during the first culture passage. (Harvey, unpubl.)

Cultivar	Division	Temperature		
		16 °C	20 °C	24 °C
King Alfred	1	231 \pm 41	198 \pm 17	258 \pm 24
Carlton	2	186 \pm 36	196 \pm 30	–
St. Keverne ^a	2	221 \pm 35	359 \pm 34	270 \pm 34
Flore Pleno	4	205 \pm 31	291 \pm 64	238 \pm 26
Hawera	5	191 \pm 11	254 \pm 28	208 \pm 25

^a Analysis of variance showed a statistically significant effect of temperature ($P < 0.05$).

in vitro and maintain vigour. However, there was no consistent effect on rooting perhaps because of the presence of BA ($4.4\mu\text{M}$) in the multiplication medium.

Declining vigour with subculturing could be more of a problem with some cultivars than others. Cultivars such as Hawera (division 5) and St. Keverne (division 2) have been multiplied for periods of up to 5 years as rapidly proliferating grass-like cultures using the methods of Chow et al. (1992b) with no noticeable reduction in their multiplication rates and no loss of vigour (unpubl.). Other cultivars (e. g. Carlton and Fortune, division 2) have proved more problematic, forming large broad leaves which may repress the production of new shoot initials even after severe cutting back and splitting.

Differences in growth and multiplication may also be influenced by treatments imposed in earlier culture passages. For example, Hussey (1982) found that leaf growth during the fourth subculture passage could be related to the concentration of growth regulators used in the initiation passage even though all the tissues had been grown on the same multiplication medium ($17.7\mu\text{M}$ BA and $0.6\mu\text{M}$ NAA) for four passages subsequently. Increasing the initiation concentrations of BA from 8.9 to $71.1\mu\text{M}$ or NAA from 1.3 to $21.5\mu\text{M}$ both resulted in increased leaf extension and a combination of the highest concentrations of both regulators gave maximum leaf extension. Such carry-over effects are surprising because multiplication of *Narcissus* seems relatively insensitive to growth regulators. Seabrook (1990) found little effect on shoot numbers when BA and NAA concentrations were varied 20-fold, the BA:NAA ratio being maintained at 10:1 (by weight). This ratio was optimal for cv. Lord Nelson (division 1). Furthermore, vigorous shoot clump cultures of cv. Hawera (division 5) appeared insensitive to both concentration and ratio of growth regulators (Harvey, unpubl.). Growth (fresh weight) and multiplication were not affected by increasing BA and NAA concentrations ten-fold or by changing the BA:NAA ratio from 10:1 by weight to either 1:1 or 100:1.

These apparently contradictory observations may simply indicate that *Narcissus* cultivars from different genetic backgrounds differ in their responses to in vitro conditions. Genotypic differences may also explain why *N. papyraceous* shoot clusters can be multiplied in liquid shake culture without hyperhydration problems (Bergoñón et al. 1992) and why these shoots can be transferred to soil directly, although bulbil formation in vitro is essential for successful transplantation of cultures of large-flowered hybrids (Hussey 1982; Seabrook 1990).

2.3 Bulbil Formation

Bulbils form freely when *Narcissus* bulb explants are incubated in vitro (Hussey 1975; Seabrook et al. 1976; Steinitz and Yahel 1982), but multiplication can be increased greatly by subculture of in vitro shoots prior to production of bulbils. This is the usual procedure for micropagation of many

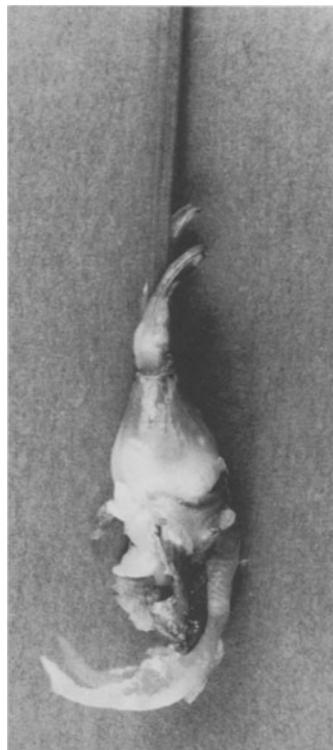
bulbous species (van Aartrijk and van der Linde 1986). However bulbils are rarely formed by shoot clump cultures growing vigorously on multiplication medium. Hussey (1976) reported that in vitro shoots eventually lost vigour, became senescent and formed bulbils. Subsequently, Squires and Langton (1990) used culture for 3 months on hormone-free medium with activated charcoal for the leaf senescence and bulbil production stage of a *Narcissus* micropropagation system.

Seabrook (1990) reported that bulbing was improved by use of a modification of Knudson's medium with Heller's microsalts and also by modifying the carbon source or the osmolarity of the medium. Inclusion of mannitol (8 or 16 g l^{-1}) in low sucrose (15 g l^{-1}) medium or substitution of glucose for sucrose "considerably improved bulbing" of *N. pseudonarcissus* cv. Lord Nelson (division 2). However, no information was presented on the rate of bulbil formation or on the size of the bulbils produced.

Rapid bulbil production from *Narcissus* shoot clump cultures can be stimulated simply by culturing on a medium with a high concentration of sucrose (Chow et al. 1992a). On transfer from multiplication medium (MS salts with 30 g l^{-1} sucrose, $0.54\mu\text{M}$ NAA and $4.44\mu\text{M}$ BA) to a hormone-free medium with 90 g l^{-1} sucrose, all cultivars tested (representatives of divisions 2, 5 and 7) formed visible bulbils within 5 weeks. After a second 5-week culture passage on fresh medium of the same composition, cultivars Saint Keverne and Hawera had formed an average of one bulbil per shoot transferred to bulbing medium. A typical bulbil produced by this method is shown in Fig. 5. Inclusion of NAA in the medium increased bulbil numbers and BA was inhibitory (Table 6). Inclusion of activated charcoal in high sucrose medium did not improve bulbing of shoot clump cultures (Staikidou 1992). This differs from the beneficial effects reported for shoot clumps cultured on media containing low concentrations of sucrose (Seabrook 1990) and for bulb explants cultured in darkness (Steinitz and Yahel 1982).

Plant growth regulator effects on bulbil initiation and growth have been explored using single leaf explants of *Narcissus*. These explants (see Sect. 2.2) are more sensitive to growth regulators than shoot clump cultures (Table 7) and rarely form bulbils unless sucrose concentration is increased above 30 g l^{-1} and auxin is included in the medium (Staikidou et al. 1994). Genotypic differences in auxin responses were demonstrated using this single leaf culture system, cv. Saint Keverne showing good bulb development with either $0.54\mu\text{M}$ NAA, $5.4\mu\text{M}$ IAA or $5.4\mu\text{M}$ IBA but cv. Hawera responding only to $27\mu\text{M}$ IAA (Staikidou et al. 1994).

The greater sensitivity of small explants to plant growth regulators and the genotypic variation in responses revealed by the use of single leaf explants may explain apparent contradictions in the literature about requirements for bulbil formation. The demonstration of genotypic differences in bulbing responses also indicates that it may be possible to improve bulbing of individual cultivars by optimising the growth regulator supply. Indeed divisions of *Narcissus* which originate from different species may differ in their requirements for bulbil initiation and development in vitro. However, Chow (unpubl.) observed that shoot clump cultures of diverse genotypes all responded satisfac-



5



6

Fig. 5. Bulbils of cv. Hawera (division 5) produced 10 weeks after transferring shoot clump cultures to agar-solidified bulbing medium. Actual bulbil diameter 4.5 mm. (Harvey, unpubl.)

Fig. 6. Bulbils of cv. Hawera (division 5) produced 6 weeks after transferring shoot clump cultures to liquid bulbing medium. Actual bulbil diameter 10 mm. (Harvey, unpubl.)

Table 6. The number of bulbils formed per shoot transferred, and width of the largest bulbil for shoot clump cultures of *Narcissus* incubated on MS medium supplemented with different combinations of sucrose, BA ($4.44 \mu\text{M}$) and NAA ($0.54 \mu\text{M}$). The data presented are for the end of the second culture passage and are the means of two cultivars, Hawera and St. Keverne. (After Chow et al. 1992a)

NAA	BA	Bulbil number			Bulbil width (mm)		
		Sucrose concentration (g l^{-1})			Sucrose concentration (g l^{-1})		
		30	90	Mean*	30	90	Mean
+	+	0.08	0.90	0.49 ^{ab}	1.37	4.54	2.96 ^{ab}
+	-	0.30	1.32	0.81 ^c	2.88	5.75	4.32 ^c
-	+	0.01	0.70	0.36 ^a	0.22	4.40	2.31 ^a
-	-	0.20	1.02	0.61 ^b	1.90	6.35	4.13 ^{bc}
Mean		0.15	0.98		1.59	5.26	

Significant effects on bulbil number per shoot transferred: sucrose concentration $P < 0.001$; growth regulator $P < 0.001$; cultivar and all interactions not significant.

Significant effects on bulbil width: sucrose concentration $P < 0.001$; growth regulator $P < 0.01$; cultivar $P < 0.05$; all interactions not significant.

*Values within a column which differ significantly ($P < 0.05$) have no superscripts in common.

Table 7. Bulbil formation by single leaf and shoot clump cultures: effects of NAA ($0.54\mu\text{M}$) and BA ($4.44\mu\text{M}$) after two culture passages on media containing 90g l^{-1} sucrose. The results are the means of cultivars St. Keverne and Hawera. Numbers are square root transformed. (Modified from Staikidou et al. 1994)

NAA	BA	Bulbil number	Bulbils dry wt. (mg)
Single leaf cultures			
—	—	0.15	1.7
+	—	0.86	11.5
—	+	0.00	0.0
+	+	0.10	0.9
SE ^a		0.092	1.72
P ^a		0.002	0.012
Shoot clump cultures			
—	—	1.93	82.5
+	—	2.41	178.4
—	+	1.77	56.8
+	+	1.90	115.3
SE ^a		0.137	15.73
P ^a		NS	NS

^aFor the interaction between NAA and BA.

rily when cultured on his standard bulbing medium (MS supplemented with 90g l^{-1} sucrose and $0.54\mu\text{M}$ NAA, solidified with agar).

Although use of high sucrose medium containing auxin results in rapid bulbing of shoot clump cultures, the bulbils are little bigger than those produced spontaneously after shoot senescence. Unsuccessful attempts have been made (Harvey, unpubl.) to increase bulbil size by changing the basal medium, for example to BDS medium developed for tissue culture of *Allium cepa* (Dunstan and Short 1977). It had been hoped that BDS medium would be more favourable for bulbing than MS basal medium (which has high concentrations of nitrate and ammonium) because fertiliser recommendations for *Narcissus* state that the crop may be harmed by high levels of nitrogen (ADAS 1984). Varying temperature in the range of 16–24°C also had no significant effect on bulbing of shoot clump cultures grown on high-sucrose medium, consistent with the lack of major effect of temperature on fresh weight accumulation of cultures grown on 30g l^{-1} sucrose multiplication medium (Table 5).

Staikidou (1992) made a careful study of the effects of carbon source and osmotic potential of the medium on bulbil formation by shoot clump cultures of the cultivars Hawera and St. Keverne. Neither bulbil initiation nor bulbil growth was improved by modifying the carbon source and concentration (60, 90 or 120g l^{-1} supplied as glucose, fructose, glucose + fructose, compared to 90g l^{-1} sucrose), or by modifying the osmotic potential of the medium (by adding mannitol or sorbitol while maintaining sucrose concentration at 30g l^{-1}). This appears to contradict Seabrook's report (1990) that bulbing was

improved by such modifications. The discrepancy is not difficult to explain: Seabrook compared the treatments with a control medium containing only 15 g l^{-1} sucrose which is unfavourable for bulbing, whereas Staikidou compared the treatments with controls cultured on medium containing 90 g l^{-1} sucrose which stimulates rapid bulbing.

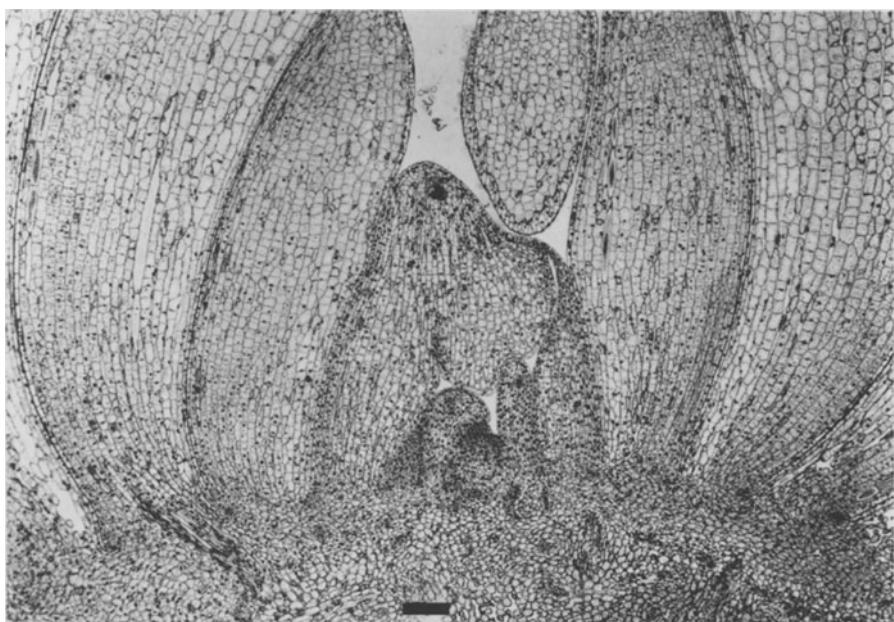
Liquid culture techniques are also being investigated. "Feeding" bulbils initiated on solidified bulbing medium by repeated addition of liquid bulbing medium (cf. the liquid medium additions technique described by Maene and Debergh 1985) has had variable effects but transfer of small shoot clumps from multiplication medium to gently shaken liquid bulbing medium has given promising preliminary results (Harvey, unpubl.). Visible bulbils appear within 2 weeks. Some of these grow rapidly and weigh up to 1 g (fresh weight) after only 6 weeks (Fig. 6). Light microscopy shows that the arrangement of bulb scales, basal plate and root primordia is similar to that described for the developing bulbs of *Narcissus* seedlings (Chan 1952). Numerous developing leaves and primordia are evident in the centre of the bulbil (Fig. 7) instead of a distinctive apical bud, but Stant (1952) presented a micrograph showing similar complexity at the shoot apex of a normal bulb of *N. pseudonarcissus*.

Growth potential of these *Narcissus* bulbils has not yet been fully assessed but liquid culture systems have been used successfully for the production of *Gladiolus* corms (Ziv 1989; Steinitz et al. 1991) and lily bulbs (van Aartrijk et al 1990). Ziv (1989) considers that storage organ formation is less susceptible to hyperhydration in liquid culture than normal shoot growth. Indeed *Narcissus* cultivars tested of divisions 1, 2, 4 and 5 all respond well to culture in liquid bulbing medium (Harvey, unpubl.) but further knowledge of the regulation of bulbil initiation and development is needed to enable control of the numbers, size and physiological state (e.g. dormancy, see Sect. 2.4) of the bulbils produced.

2.4 Growth Ex Vitrium

In vitro bulbils formed directly from *N. tazetta* bulb explants could be transplanted to the field successfully if they were 250–300 mg fresh weight and had two leaves and an abundant root system (Steinitz and Yahel 1982). No dormancy problems were reported with these bulbils formed directly from bulb explants. However, bulbils are generally dormant when produced by allowing shoot clump cultures to senesce. A dormancy-breaking treatment is then required before planting out as, despite fungicide treatment, dormant bulbils tend to rot unless planted in sterile growing media (Hussey 1982).

Dormancy can be broken simply by storage of cultures in darkness at $5\text{ }^{\circ}\text{C}$ until shoots begin to emerge. Using the shoot senescence method of bulbil production and a cold treatment for dormancy-breaking, Squires and Langton (1990) identified bulbil weight as an important factor determining transplantation success. Subsequently, a large-scale glasshouse trial of the same system (Squires et al. 1991) demonstrated that the best transplantation success was



A

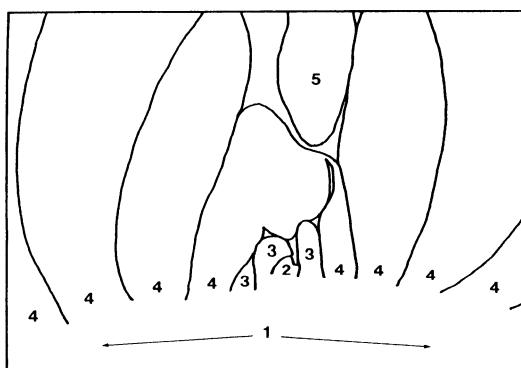


Diagram of LS through centre of bulbil

- 1 Basal plate
- 2 Primordia
- 3 Developing leaf or leaf scale
- 4 Base of leaf or leaf scale
- 5 Leaf in tangential section

B

Fig. 7. Longitudinal section of cv. Hawera (division 5) bulbil produced in liquid culture. **A** Micrograph (scale bar 100 μ m); **B** labelled diagram. (Fraser and Harvey, unpubl.)

obtained using bulbils weighing more than 0.2 g fresh weight. Unfortunately, the overall proportion of such bulbils was low (19–20%). Increasing bulbil weight by increasing sucrose levels during the shoot senescence phase did not improve transplantation success, possibly due to increased dormancy. Bulbil production by culturing of senescent shoots in darkness also reduced transplantation success, again apparently due to increased dormancy. The authors stated that “most unsuccessfully transplanted bulbils showed a foliar growth phase suggesting a pre-mature re-entry into dormancy”.

Bulbil dormancy can also be broken by gibberellin treatment (Seabrook 1990), but growth after transplantation has not been reported.

No dormancy problems have been encountered on transplantation of bulbils produced by transferring actively growing shoot clump cultures to solidified medium containing auxin and a high concentration of sucrose (Chow et al. 1992a). Leaf elongation and leaf production continued when these bulbils were transplanted into compost and grown with a 15-h photoperiod at 12°C and 9-h dark period at 9°C (Table 8). After 2 months under these conditions, the plantlets were transferred to an unheated glasshouse (autumn 1990) and cv. Hawera flowered 18 months later (spring 1992). St. Keverne and other large-flowered cultivars flowered the following year. All flowers were true to type.

Transplantation success of large bulbils produced in liquid culture has yet to be evaluated. Problems may be encountered because root development is poor in liquid medium (Fig. 6). However, some liquid culture bulbils of cv. St. Keverne and Carlton planted in an unheated glasshouse in late 1993 showed vigorous leaf growth in spring 1994 and doubled their weight over the growing season. Large-scale trials of a wider range of cultivars will be needed. It is possible that these large bulbils may withstand direct planting into field conditions and may also reach flowering size more quickly than the small bulbils produced using solidified culture media.

Table 8. Effects of sucrose supply and growth regulators on bulbil formation in vitro and on growth 8 weeks after planting in compost. (Modified from Chow et al. 1992a)

Effects	End of second culture passage		8 weeks after planting out	
	Width of largest bulbil (mm)	Length of longest leaf (mm)	Length of longest leaf (mm)	
Sucrose concentration:				
30 g l ⁻¹	1.59	80	105	
90 g l ⁻¹	5.26	25	86	
Growth regulators				
NAA (0.54 µM)	BA (4.44 µM)			
+	+	2.96	52	75
+	-	4.32	55	123
-	+	2.31	46	75
-	-	4.13	57	110
Cultivar:				
Hawera	2.94	64	113	
St. Keverne	3.92	41	78	

Significant effects and interactions at end of second culture passage. Bulbil width: sucrose $P < 0.001$; growth regulator $P < 0.01$; cultivar $P < 0.05$; all interactions not significant. Leaf length: sucrose $P < 0.001$; cultivar $P < 0.001$; growth regulators and all interactions not significant.

Significant effects and interactions at eight weeks after planting out. Sucrose $P < 0.001$; growth regulator $P < 0.001$; cultivar $P < 0.001$; all interactions not significant.

2.5 Commercial Micropagation

The low multiplication rates of *Narcissus* in vitro and the need for a bulbing phase mean that micropagation is a slow and laborious process. At present any commercial micropagation is on a small scale, limited to special orders for early multiplication of particularly valuable new cultivars (van der Linde, pers. comm). Subsequent multiplication is by chipping (see Sect. 1.2).

To be successful on large scale, commercial micropagation would have to give better multiplication rates than chipping or twin scaling without any substantial difference in costs. This would be difficult to achieve because mechanisation of chipping has greatly reduced labour requirements. Greatly improved in vitro culture techniques for both multiplication of shoot clump cultures and for bulbil formation would be needed.

3 Summary and Conclusions

This chapter reviews the literature on *Narcissus* which reveals many difficulties with initiation of cultures, low multiplication rates, slow bulbil formation and poor success rates for transplantation to soil. Recent progress towards solving these problems is discussed and a protocol is presented for micropagation and transfer of in vitro bulbils to soil. Although reliable, this method is unlikely to replace chipping as a rapid and inexpensive method of bulking up commercial stocks of *Narcissus*. Nevertheless, micropagation is important as a first stage in multiplication of valuable bulbs.

Two major problems remain:

1. Multiplication rates are very low for some cultivars, apparently due to very strong apical dominance in shoot clump cultures. Multiplication is improved somewhat by physical treatments designed to break apical dominance but these treatments are laborious.
2. Large non-dormant bulbils are needed for successful transfer to soil. Methods relying on spontaneous bulbing of senescent shoots are slow and produce small bulbils which require a dormancy-breaking treatment before planting out. Transplantation success is poor. A technique for much more rapid production of non-dormant bulbils has been developed, but the bulbils are no larger than those produced by shoot senescence. Liquid culture techniques for rapid production of large bulbils seem promising but performance of these bulbils in soil has not yet been fully assessed.

Cultivar differences in response to in vitro culture are also problematic. It seems that different techniques may be needed for cultivars from different genetic backgrounds. However, the classification of *Narcissus* is so complex and hybridisation has been so extensive that the origins of commercial cultivars are often difficult to trace.

In conclusion, development of in vitro techniques suitable for commercial large-scale micropropagation of *Narcissus* remains a challenge.

An important future role of in vitro culture of *Narcissus* seems likely to be in molecular biology, for introduction of genes for improved pest and disease resistance and perhaps also improved flower quality and enhanced production of secondary metabolites. Micropropagation would have a special role in the early multiplication of improved genotypes, producing enough bulbs for subsequent propagation by chipping.

4 Protocol

Plant Material. Bulbs should be in a non-dormant condition but not in active growth, to permit hot-water treatment.

Initiation. Store bulbs at 30°C for at least 4 weeks to pre-condition for hot-water treatment. Trim roots down to the level of the base plate and remove outer dried scales. Immerse bulbs in hot water (54°C) for 1h. Cut away the apical third of the bulb and cut the basal portion longitudinally into half. Surface-sterilize the bulb section for 30 min in 1% (w/v) sodium hypochlorite, then rinse three times in sterile water.

Cut away the tissue damaged by the sterilant from the base plate and cut the apical part of the leaves and scales to approximately 10mm long. Divide the remaining tissue into 4–8 “mini chip” explants (depending on bulb size) by making radial cuts. Inoculate explants with the basal end in MS medium with 30 g l⁻¹ sucrose, 0.54 µM NAA, 4.44 µM BA and 7 g l⁻¹ agar, pH 5.6 until shoots and bulbils are visible (about 5 weeks). Incubate at 20°C with a 16-h photoperiod (65–75 µmol m⁻² s⁻¹ PAR at bench height) for a further 5 weeks.

Multiplication. After initiation as described above, cut out any bulbils and young developing leaves making sure to leave a section of bulb base plate attached. Transfer these to the same conditions as used for the second initiation passage. Use a 5-week subculture regime where at each transfer shoot clumps are divided longitudinally so that leaves attached to base plate tissue are transferred. Tissues approximately 10mm in diameter with 2–8 leaves are used to inoculate cultures. Use alternate severe leaf cutting (i.e. all leaves cut down to the base plate) and recovery (no leaf cutting) culture passages to maintain vigour and stimulate leaf production. If bulbils develop, they should be split longitudinally.

Bulbil Production. Split shoot clumps into pieces approximately 10mm in diameter, transfer to bulbing medium (MS with 90 g l⁻¹ sucrose, 0.54 µM NAA and 7 g l⁻¹ agar, pH 5.6) and culture in the same environmental conditions as for initiation and multiplication. Transfer to fresh medium after 5 weeks.

Transfer to Soil. After two 5-week passages on bulbing medium, split clumps to separate the larger bulbils and soak in fungicide (carbendazim, 1.125 g l⁻¹). Plant in a compost: perlite mixture (2:1) treated with fungicides (0.15 g tolclofos-methyl and 0.386 g propamocarb hydrochloride in 500 ml water, applied to 10 l growing medium). Grow initially under cool, long-day conditions (e.g. 15-h photoperiod at 12°C, 9-h dark period at 9°C).

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I.18 Micropropagation of *Otacanthus* Species

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1 Introduction

1.1 Botany, Distribution, and Importance

The genus *Otacanthus* (family Scrophulariaceae) consists of six described species native to eastern Brazil. They grow in diverse habitats, such as coastal sand dunes (restinga) or shady rock slopes in Atlantic rain forest, from 0 to more than 2500 m elevation. *Otacanthus coeruleus* is found naturalized in the north of Brazil, in Madagascar, La Réunion, and the Seychelles. All species of *Otacanthus* consist of subshrubs with simple, opposite leaves and showy, purple to blue flowers with a white spot on the lower lip. A nearly albino form of *O. platychilus* has also been found. The flowers occur solitary in the axil of the upper leaves. In nature, there does not appear to be a definite flowering period (Ronse 1993; Ronse and Philcox 1993).

Otacanthus coeruleus is the species with the largest flowers. Besides its use as an ornamental in Brazil (Taubert 1890; Billiet 1982) and French Guiana (Billiet 1991), there are records of its cultivation as an ornamental pot plant during last century in France and Belgium (Van Houtte 1862); this culture disappeared. This species has been reintroduced into cultivation more recently and is now grown as a garden plant and for cut flowers in Australia (Billiet 1982, 1991). A recent Danish study has shown its potential as a cut-flower crop (Geertsen 1990). Other species might also possess value as ornamentals, such as *O. platychilus*, as was already pointed out by Radlkofer (1885).

Otacanthus species might also have some economic value because of their essential oils. Several species were found to contain the novel sesquiterpene β -copaen-4 α -ol, and *O. coeruleus* contains high amounts of α -copaene (De Pooter et al. 1989; Ronse 1993). This compound is used as a pheromone in the control of *Ceratitis capitata*, the Mediterranean fruit fly (Jacobson et al. 1987).

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1.2 Conventional Propagation and Need for Micropagation

As already mentioned by Van Houtte in 1862, *O. coeruleus* is easy to grow in a warm greenhouse. It can be grown in several mixtures based on peat or leaf mold, or in a standard potting medium. The substrate should not be too alkaline, as yellowing of the top leaves is frequently observed, possibly due to iron deficiency. All *Otacanthus* species seem to prefer a soil with an acidic reaction. They need a fair amount of watering, and they can flower at any time of the year. In order to obtain well-branched plants, pinching out once or twice will be necessary. The flowering period lasts at least 5 months; after the flowering period the plants are pruned back severely.

The main pest on *Otacanthus* in culture is the whitefly, *Trialeurodes vaporariorum*. Plants of *O. fluminensis* collected in their natural habitat were attacked by root-knot nematodes (*Meloidogyne* sp.).

Most species of *Otacanthus* are easily propagated by cuttings and by sowing. Stem cuttings of *O. coeruleus* treated with a commercial rooting preparation, containing 0.5% IAA, will root within 2 to 3 weeks when placed in peat on a heated bench in a greenhouse. Depending on the time of the year, flowering plants can be obtained within 4 to 6 months.

All species can also be sown. About 8 weeks after self-pollination, many tiny seeds are obtained. Fresh seeds sown in the warm greenhouse (day/night temperature 24/20°C) germinate within 1 to 2 weeks.

Although conventional propagation of *Otacanthus* is rather easy, micropagation yields more plants in a shorter time. We have used it for rapid multiplication of some plants with interesting features, e.g. large flowers. In vitro culture methods have also been devised mainly for the influence of environmental factors on essential oil production in *Otacanthus*. (Ronse et al. 1997).

2 Micropagation and In Vitro Studies

The experiments in our laboratory are the first in vitro studies carried out on *Otacanthus* spp. We have worked out a micropagation procedure for *O. coeruleus*, and investigated the influence of several environmental factors on the in vitro morphogenesis and growth of this species. In addition, we have investigated the micropagation of two other species, *O. platychilus* (Radlk.) Taub. and *O. villosus* Philcox, and the production of essential oils in vitro by *O. coeruleus*.

2.1 Micropagation of *Otacanthus coeruleus*

The basic propagation techniques have been described by Ronse and De Proft (1992). Leaf pieces and nodal stem segments were put on a basal medium supplemented with combinations of NAA, BA and 2, 4-D. The basal medium

consists of the macroelements and the iron-EDTA solution of MS (Murashige and Skoog 1962), the microelements and vitamins of Nitsch and Nitsch (1969), with 6 g/l agar and 25 g/l sucrose.

All leaf explants died except those with 5 μ M NAA + 2.2 μ M BA, which produced callus. Stem explants showed more response (see Table 1 and Fig. 1). Callus formation was induced with several hormone treatments, especially with 5 μ M NAA + 2.2 μ M BA, and elongation of axillary buds without formation of adventitious buds also occurred. Formation of adventitious buds and shoot proliferation were stimulated by 0.5 μ M NAA + 0.44 μ M BA, with 0.5 μ M NAA + 2.2 μ M BA, and with 0.5 μ M NAA + 4.4 μ M BA. With the third treatment proliferation was only obtained in the second replication, but it took place at a high rate, whereas it was very slow for 0.5 μ M NAA + 0.44 μ M BA. With 0.5 μ M NAA + 2.2 μ M BA proliferation was the most rapid, yielding six new clumps every 2 weeks for every clump of proliferating shoots.

Calluses placed on Monnier's medium (Monnier 1976) produced shoots. Shoots that were obtained from callus or from direct proliferation, were easily rooted on hormone-free medium and 1 g/l charcoal (100% rooting after 2 months). Plantlets were successfully transferred from in vitro conditions to the greenhouse (80% survival) and flowered 10 months later (Fig. 2).

2.2 Influence of Environmental Factors on In Vitro Growth and Morphogenesis of *O. coeruleus*

The factors that were tested are: light intensity, temperature, macro-nutrient content, agar content, and sucrose concentration, either with or without addi-

Table 1. Organogenetic response (in percentage of explants) of nodal stem segments of *O. coeruleus* to various types and concentrations of plant growth regulators. (After Ronse and De Proft 1992)

Plant growth regulators (μ M)	Callus formation		Outgrowth of nodal buds		Shoot proliferation ^a	
	R1	R2	R1	R2	R1	R2
-	0	0	9	12	0	0
NAA 0.5	0	50	43	75	0	25
NAA 0.5 + BA 0.44	43	86	43	86	43	86
NAA 0.5 + BA 2.2	6	50	24	20	35	80
NAA 0.5 + BA 4.4	100	17	0	0	0	67
NAA 5.0 + BA 2.2	88	100	0	0	0	29
BA 2.2	0	43	0	43	0	57
BA 13.2	0	0	0	0	0	0
2,4-D 0.5	37	43	5	43	0	29
2,4-D 2.5	0	100	0	0	0	0

The results of two replications are given, consisting of 12 and 10 explants, respectively. R1 and R2, percentage of explants with a particular response in replication 1 and 2, respectively.

^aShoots formed from adventitious buds.

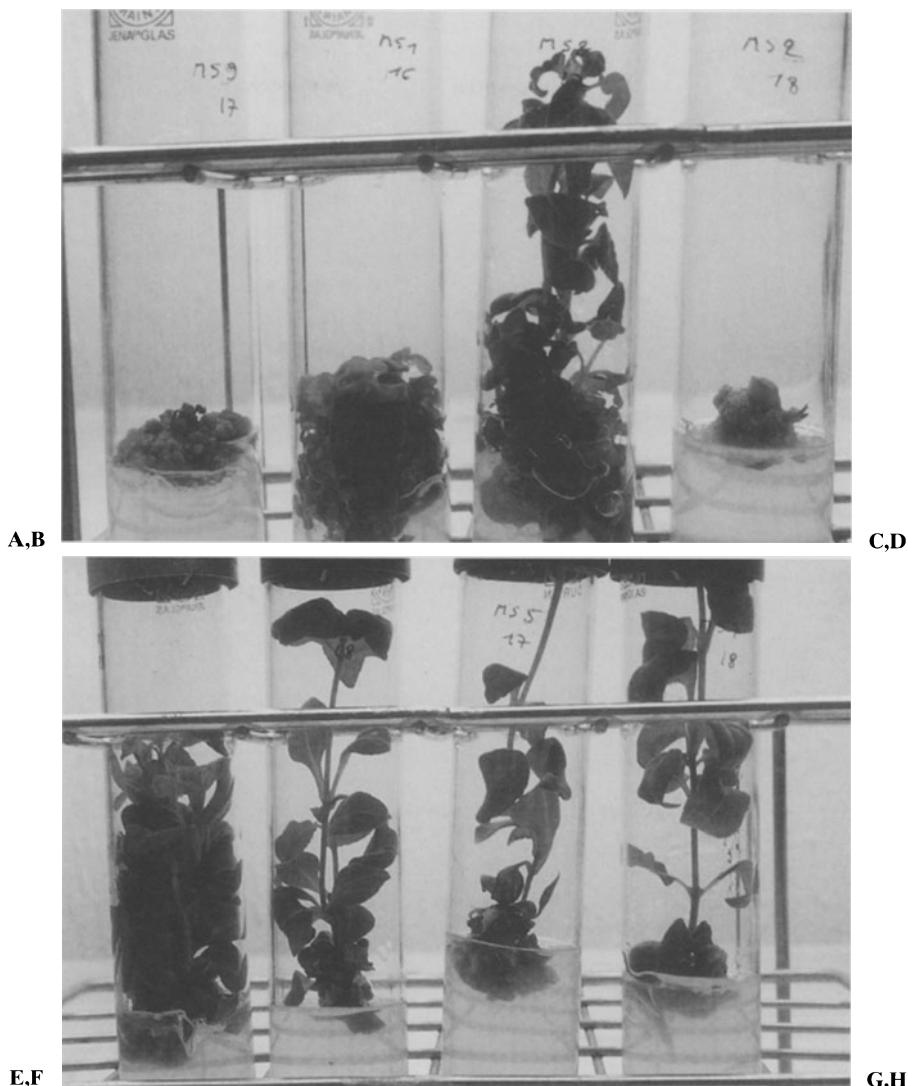


Fig. 1A–H. Nodal stem segments after 3-month initiation with different PGR treatments (in μM): **A** 2.5 BA + 5 NAA; **B** 4.4 BA + 0.5 NAA; **C** 2.2 BA; **D** 2.5 2,4-D; **E** 2.2 BA + 0.5 NAA; **F** 0.5 NAA; **G** 0.5 2,4-D; **H** 0.44 BA + 0.5 NAA

tion of mannitol (Ronse and De Proft, in preparation). Mannitol was added in amounts so as to obtain a constant osmotic potential of -3.5 bar (equivalent to that of the same medium with 50 g/l sucrose).

It was observed that the growth of proliferating cultures was better with $70 \mu\text{mol m}^{-2}\text{s}^{-1}$ and at $25/20^\circ\text{C}$ than with $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ at $20/15^\circ\text{C}$. The growth as a function of the other factors is represented in Fig. 3; it is expressed by the relative growth index (RGI: the increase of growth after one subculture,



Fig. 2. Flowering plants of *O. coeruleus* obtained from micropropagation

Table 2. Mortality, fresh weight and relative growth index (RGI) of proliferating shoot cultures as function of sucrose and mannitol concentration

Sucrose (g/l)	Mannitol (g/l)	Mortality (%)	Weight (g)	RGI
5	0	46	16.2	0.97
	24.0	0	51.2	1.37
10	0	8	34.9	1.52
	21.3	0	42.0	1.64
20	0	8	60.6	2.23
	16.0	0	75.9	2.45
25	0	0	55.2	2.11
	13.3	4	84.7	2.38
30	0	4	83.2	2.19
	10.7	0	80.8	2.85
40	0	4	57.4	1.33
	5.3	0	93.3	2.49
50	0	21	36.7	1.16

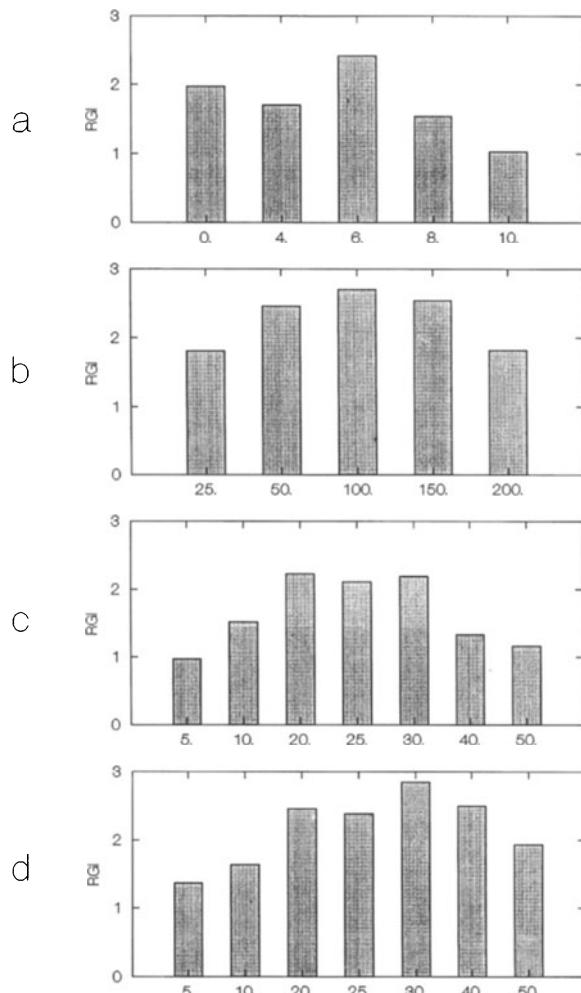


Fig. 3a-d. Effect of medium composition on the growth (RGI) of proliferating shoot cultures of *O. coeruleus*: **a** agar concentration (g/l); **b** macrosalt concentration (in %); **c** sucrose concentration (g/l); **d** sucrose concentration (g/l) with mannitol

divided by the initial weight). The optimal growth was found at agar levels of 6 and 0 g/l, at a macro-salt concentration equal to that 1 or 1.5 times that of MS, and at sucrose concentrations between 20 and 30 g/l. Moreover, the addition of mannitol enhanced the growth at most sucrose levels.

The mortality and weight of the cultures at varying sucrose concentrations, with or without addition of mannitol, are shown in Table 2. The lowest and the highest sucrose concentrations provoke a significant mortality, which does not occur when mannitol is added. Mannitol addition also has a positive effect on the growth at most sucrose concentrations.

The morphology of the explants as influenced by the nutrient medium components is shown in Fig. 4. Agar contents under 6 g/l yield fast-growing, thin and vitrified shoots (Fig. 4A). The same effect is even more marked at the two lowest macro-salt concentrations, whereas at higher concentrations the proliferation is slowed down and the leaves grow more normally (Fig. 4B).



Fig. 4A-D. Proliferating shoot cultures of *O. coeruleus* on varying concentrations of nutrient medium components: **A** agar 0–10 g/l (2 tubes per treatment); **B** macro-salt concentration 25–200% of MS; **C** sucrose 5–50 g/l; **D** sucrose 5–50 g/l with mannitol addition (see text)



Fig. 4C,D.

Low sucrose concentrations, either with or without mannitol, also yield vitrified, but highly proliferating explants (Fig. 4C,D).

The above-mentioned growth results are averages for five subcultures, but the growth can change according to the number of previous subcultures, as shown in Fig. 5 for varying sucrose concentrations. At sucrose concentrations between 5 and 20 g/l the growth decreased with an increasing number of

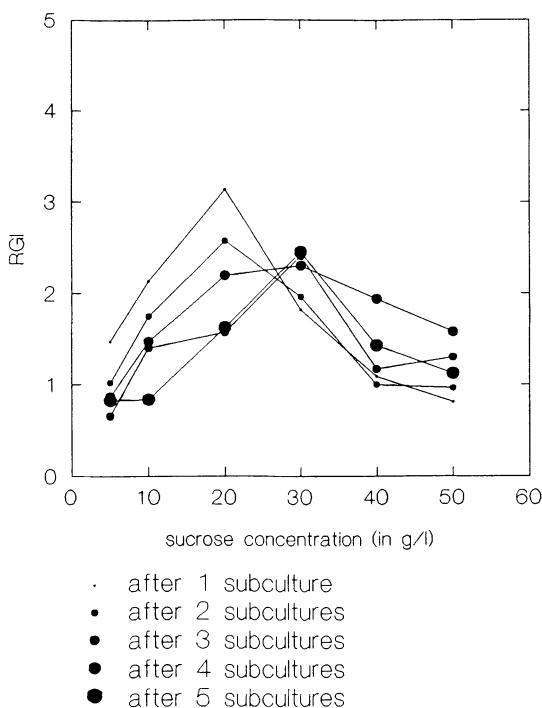


Fig. 5. Growth (RGI) of proliferating shoots of *O. coeruleus* as a function of the sucrose concentration of the medium after one to five subcultures

subcultures, but this effect was not present at higher concentrations, rather the reverse was true.

2.3 Micropropagation of *O. platychilus* and *O. villosus*

O. platychilus can also be micropropagated relatively easily. Leaf and nodal stem segments were put on the basal medium as for *O. coeruleus*, including either $0.5 \mu\text{M}$ NAA + $4.4 \mu\text{M}$ BA or $0.5 \mu\text{M}$ NAA + $2.2 \mu\text{M}$ BA. Leaf segments were additionally placed on a medium with $5 \mu\text{M}$ NAA + $2.2 \mu\text{M}$ BA. The leaf segments only responded to the last hormone concentration by developing callus, as did *O. coeruleus*. Nodal stem segments formed adventitious buds with the two treatments. For *O. platychilus* the fastest proliferation was found with $0.5 \mu\text{M}$ NAA + $4.4 \mu\text{M}$ BA, but this treatment gave a high mortality of the initial explants (42%) and some vitrification. Two months after the start of the cultures, the surviving explants could be divided into eight pieces, and those on $0.5 \mu\text{M}$ NAA + $2.2 \mu\text{M}$ BA were divided into four pieces. Rooting of shoots, which had been obtained on both media, was 100% successful after a few weeks on basal medium with 1 g/l activated charcoal.

Several trials were effected for the transfer of the plantlets to greenhouse conditions, and four substrates were tested: two peat-based, commercial substrates, and mixtures of one-half coarse sand and one-half peat or leaf mold. There was an influence of the substrate on survival, growth and flowering, but the results differed in subsequent experiments. The survival ranged between 25 and 60%; the death of explants occurred only during the first 3 weeks after transfer. After 32 weeks the plant height ranged between 33 and 62 cm, and between 20 and 60% of the plants had flowered. The earliest flowering took place 16 weeks after transfer into the greenhouse.

A form of *O. villosus*, a slow-growing species, was micropropagated in order to obtain more plants, which were needed for the extraction of an unknown sesquiterpene. The nodal stem segments all died on the basal medium with $0.5 \mu\text{M}$ NAA + $4.4 \mu\text{M}$ BA, but were successfully micropropagated with $0.5 \mu\text{M}$ NAA + $2.2 \mu\text{M}$ BA: 5 weeks after initiation the explants had increased 3.5 times. However, after several months high levels of vitrification were observed. Rooting was obtained in 50% of the cases on hormone-free basal medium supplemented with 1 g/l charcoal, but the obtained plantlets were pale and did not grow well. On basal medium without charcoal, 100% rooting and healthy plantlets were obtained.

2.4 In Vitro Production of Essential Oils in *O. coeruleus*

The content and composition of the essential oils of various in vitro cultures of *O. coeruleus*, and of the medium on which they were grown were investigated (Ronse 1993). It appears that in vitro cultures of *O. coeruleus* produce an essential oil that is similar to that of the mother plants, as described by De Pooter et al. (1989). This oil contains more than 100 mono- and sesquiterpenes, with the new sesquiterpene compound β -copaen-4 α -ol. However, the content of some individual components varies strongly with the type of in vitro culture, and so does the number of components of the oils. Moreover, in vitro cultures have an essential oil content that is about ten times lower than that of the mother plants. The media on which cultures were grown also contained significant amounts of essential oils. These quantities lie in the same range as those extracted from the cultures, but can increase up to eight times as much. The essential oils consist of mono- and sesquiterpenes, and have only one-third of their components in common with those contained in the in vitro cultures.

3 Summary and Conclusions

Most *Otacanthus* species have an ornamental potential either as pot plants or as cut flowers; they are also a source of essential oil components. *Otacanthus coeruleus*, *O. platychilus* and *O. villosus* can all be micropropagated rapidly

with similar concentrations of auxins and cytokinins. Several components of the nutrient medium influence the growth and morphology of the cultures. The optimal conditions for proliferation are similar to those for most other plant species, except that *Otacanthus* thrive on media with relatively high macro-salt concentrations. The addition of mannitol, together with sucrose, is beneficial.

In vitro cultures of *O. coeruleus* produce essential oils similar to those of the mother plants, but with contents that are about ten times lower. Varying culture conditions induce shifts into the relative amounts of oil constituents. The growing media also contain important amounts of essential oils with partly different constituents from those in the cultures.

The general conclusion is that in vitro methods constitute a rapid propagation method of *Otacanthus* species in view of their production as ornamentals, and constitute an interesting tool for the study of the physiology of their essential oil production.

4 Protocol

1. Excise nodal stem segments of about 1 cm length. Rub them with a cotton plug imbibed in ethanol; immerse for 10 min in a solution of 5% sodium hypochlorite and a few drops of Tween 20; rinse three times with sterile water.
2. Place the explants vertically (top above) on a proliferation medium containing the macro-elements and iron solution of MS, and the micro-elements and vitamins of Nitsch and Nitsch, supplemented with 6 g/l agar, 25 g/l sucrose, 0.5 μ M NAA and either 2.2 or 4.4 μ M BA, depending on the species.
3. Cultures in 25 \times 150 mm tubes are incubated in a 16-h photoperiod with a photon flux of 50 to 70 μ mol s^{-1} m^{-2} at 25/20°C.
4. Cultures are transplanted on new medium every 3 weeks; at the same time clumps of proliferating shoots are divided into pieces of about 1 cm².
5. Rooting of the cultures is obtained within about 4 weeks on the basal medium without plant growth regulators and, depending on the species, with or without 1 g/l charcoal.
6. Rooted shoots are transplanted to the warm greenhouse in 6 cm pots in a commercial potting mixture; they are placed in 50% shade under glass cover, which is gradually removed to accustom the plants to a lower degree of air humidity.

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I.19 Micropropagation of *Beaucarnea recurvata* Lem. syn. *Nolina recurvata* (Lem.) Hemsl. (Ponytail Palm)

G. SAMYN¹

1 General Account

Ponytail palm (Liliaceae) is indigenous to desert regions of Texas and Mexico. The taxonomic situation can still today be assumed as confusing. Ponytail palm is well known as *Beaucarnea recurvata*, but in several references it is also classified as *Nolina recurvata*.

The genus *Beaucarnea* was constituted in 1861 by Charles Lemaire with plants, of a certain ornamental value and already cultivated until then as *Pincenectitia*. The name was given in honour of Jean-Baptiste Beaucarne, one of Belgium's famous plant collectors in the 19th century.

Baker (1873) mentioned eight species, some of which were also already in culture at that time. Later, Bentham and Hooker placed these plants in the genus *Nolina* Michx., dedicated to P.C. Nolin, a French botanist from the 18th century. However, Everett (1981) made a separation between the genus *Beaucarnea* (ponytail palm) with 12 species, and the genus *Nolina* (bear-grass) with 25 species.

Ponytail palms are very slow growers, forming straight stems which, like many of the Yuccas, extend and increase in height as they become denuded of leaves. Stems easily attain 10 m. The tall trunks are usually swollen at the base and are crowned with tufts of long and evergreen leaves.

In its native habitat at flowering time, each stem produces a terminal inflorescence more than 1 m long and broad, composed of profuse small white flowers. The flowers have six perianth segments and stamens and fruits are ordinary three-winged dry capsules.

Ponytail palm, and perhaps other *Beaucarnea* spp. may have two uses in horticulture. In temperate climates, where the winters have frost risks, they are limited to indoors or cold conservatories. In warmer regions, they are adapted for outdoor gardening or can be grown as container plants for indoor and outdoor decoration.

In the literature there is hardly any data on ponytail palm culture. Advice for growing under room conditions or in private glasshouses was already given by Baines (1885). At that time, different *Beaucarnea* species were kept for

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their ornamental value and for their rather unusual form, mainly caused by the swollen base of the stem.

This advice was similar to the observations made by Wang and Sauls (1988) a century later. Plants in commercial culture and those which are already being marketed have the following growth characteristics:

1. Ponytail palms are sun-loving plants and stay in optimal condition in locations where they get full light intensity. This is very important during commercial culture for fast growth. Under room conditions ponytail palm is, however, very tolerant to shade.
2. They should be grown in media with good aeration, as they cannot bear stagnant soil conditions, which can cause rotting of the roots.
3. Frequent irrigation with short drying cycles significantly increases growth.
4. Intermittent fertilization provides adequate nutrition, although ponytail palm seems to tolerate high levels of salts and consequently an increased electrical conductivity of the substrate.

Although the above conditions may be required for good profits under commercial culture, under room conditions ponytail palm is one of the least demanding indoor plants. Then, this slow-growing plant is resistant to very dry conditions, low relative humidity, and heavy shade, and conserves its remarkable longevity indoors. However, growth is then slower or may even completely stop.

For many years, ponytail palm was imported to Europe from its native regions in the form of whole stems or stem pieces which produce rather expensive plants, and which cannot be the basis of a system more oriented to mass production.

More recently, young plants have been cultured from seeds, since several international firms specializing in "rare plants" can now offer seeds for commercial culture. The germination percentage of seed batches can be quite good, which guarantees a problemless start to mass production. During the first months, growth is very slow and the first signs of the protuberance of the stem appear after 6 months. From that moment the small plants can be potted with the tuber just above the soil.

In this way, sexual propagation results in a plant with only one shoot apex, which can be marketed after 1 to 2 years. Multiple shoot plants with at least three shoots meet higher quality standards than seedlings with only one shoot. In vitro culture can be the solution for commercial production of such plants.

2 Micropagation

2.1 Induction of Cultures

In vitro culture can be started with 1- to 2-mm shoot apices from young seedlings germinated in peat. After excision, each apex is disinfected for

10 min in a bleach solution (1% NaOCl (w/v)], rinsed twice for 1 min in sterile distilled water, and aseptically cultured on MS medium (Murashige and Skoog 1962) containing inorganic salts and supplemented with (mg/l) *myo*-inositol, 100; nicotinic acid, 1; thiamine-HCl, 0.5; pyridoxine-HCl, 0.5; and glycine, 4. The medium is solidified with 6 g MC 29 agar/l (Lab M, Topley House, UK).

Before autoclaving at 120°C for 20 min, growth regulators are added to the medium and the pH is adjusted to 5.8. Cultures are maintained at 22 ± 1°C with a 16-h photoperiod of 75 $\mu\text{mol s}^{-1} \text{m}^{-2}$ provided by cool-white fluorescent lamps.

2.2 Propagation

Sufficiently developed (1 cm) explants are transferred after 3 months to a multiplication medium (MM) consisting of inorganic salts supplemented with

Table 1. Composition of multiplication and rooting medium for *Beaucarnea recurvata*

Components	Micropropagation (mg l ⁻¹)	Rooting (mg l ⁻¹)
Macronutrients		
NH ₄ NO ₃	400	550
KNO ₃	1800	630
CaCl ₂ .2H ₂ O	—	150
Ca(NO ₃) ₂ .4H ₂ O	1200	—
MgSO ₄ .7H ₂ O	360	120
KH ₂ PO ₄	270	60
Fe-chelate (9% Fe)	50	20
Micronutrients		
H ₃ BO ₄	6.2	2.06
MnSO ₄ .4H ₂ O	22.3	7.43
ZnSO ₄ .7H ₂ O	8.6	2.86
KI	0.83	0.27
Na ₂ M ₀ O ₄ .2H ₂ O	0.25	0.08
CuSO ₄ .5H ₂ O	0.025	0.08
CoCl ₂ .6H ₂ O	0.025	0.08
Vitamins		
Myo-inositol	50	100
Nicotinic acid	0.5	1
Thiamine-HCl	0.25	0.5
Pyridoxine HCl	0.25	0.5
Amino acid:Glycine	2	4
Auxin:NAA	—	0.250
Cytokinin:BAP	0.500	—
Gibberellin:GA ₃	0.1	—
Sucrose	20000	20000
Agar	6000	6000
pH	5.8	5.8

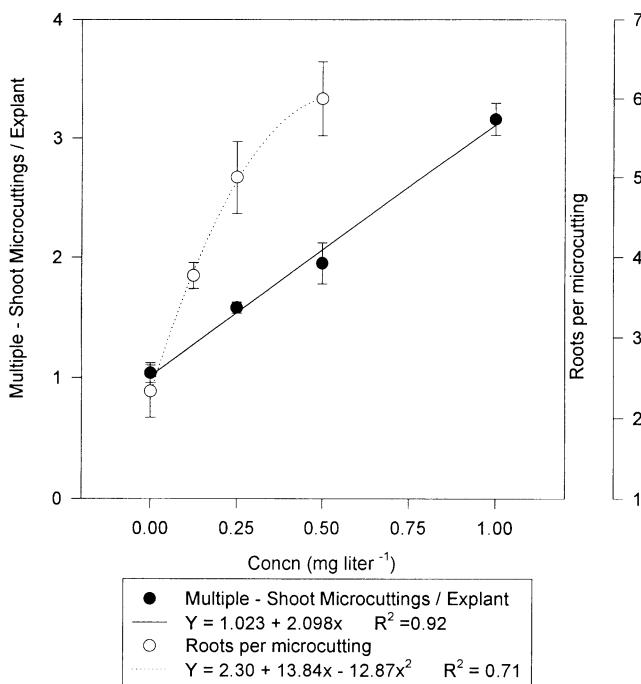


Fig. 1. Influence of BA (●) on the formation of rootable multiple-shoot microcuttings of ponytail palm and of NAA (○) on rooting the microcuttings. Regression analysis was performed with the means for each concentration of five BA or six NAA vessels with ten explants per vessel. Vertical bars equal ± 1 SE (Samyn 1993)

(mg/l) *myo*-inositol, 50; nicotinic acid, 0.5; thiamine-HCl, 0.25; pyridoxine-HCl, 0.25; glycine, 2; *N*-(phenylmethyl)-1*H*-purine-6-amine (BA), 0.5; gibberellic acid (GA₃), 0.1; sucrose, 20000; and agar, 6000, at pH 5.8 (Table 1; Quoirin et al. 1977). Explants multiply well on this medium and form many shoots on a fast-growing, tuber-like body.

This tuber-like body is an in vitro development of the swollen stem base typical in older plants, whether growing in their natural habitat, or cultured from seeds in containers for use indoors.

Because of the positive effects of BA, further experiments were performed to determine the optimal BA concentration for rapid propagation. MM was supplemented with BA at 0, 0.25, 0.5 or 1 mg/l (0, 1.1, 2.2, or 4.4 μ M BA). For each concentration, five vessels with ten explants per vessel were used. Each explant consisted of a piece of the tuber-like body with three clearly developed shoot initials. After 3 months, multiplication was determined by counting the number of explants with at least three axillary shoots, which were obtained after dividing the tuber-like body and which were suited for transfer to a rooting medium. No shoot formation resulted without BA in the medium (Fig. 1). Multiplication obtained with 1 mg/l BA

is of practical interest but is limiting, since some of the newly formed leaves remained small and were sometimes distorted. It also becomes difficult to divide the tuber-like body into explants suitable for transfer to rooting medium.

2.3 Rooting

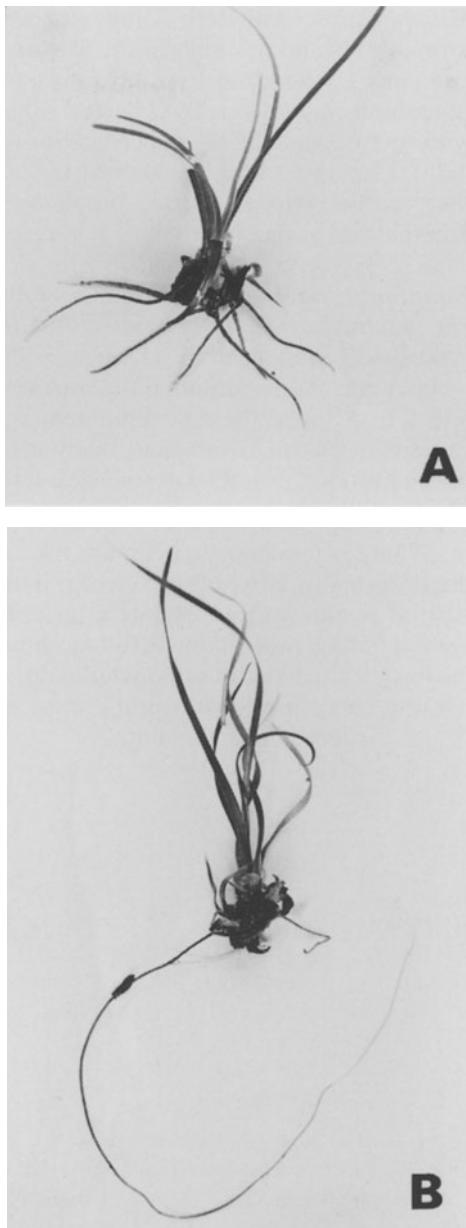
In the micropropagation system BA inhibited root induction and root growth completely. However, rooting occurred easily on one-third-strength MS medium without growth regulators, but the number of roots was increased by 1-naphthaleneacetic acid (NAA; Table 1). The influence of NAA was determined on one-third-strength MS medium supplemented with NAA at 0, 0.125, 0.250, or 0.5 mg/l (0, 0.67, 1.34, or 2.68 μ M NAA). For each concentration, six vessels with ten explants from MM per vessel were used. Roots were initiated quickly, and they developed well with NAA at 0.125 and 0.250 mg/l (Fig. 1). A concentration of 0.5 mg/l was too high and caused leaf distortion. Roots formed in the absence of NAA and those induced by NAA developed differently. Without NAA, one or two root initials per explant appeared after 3 to 4 weeks, and each became a fine, very long, branched root. In the presence of NAA, several initials appeared after 2 weeks and remained straight (Fig. 2). During the second month, some of the roots started branching. Although in vitro root initiation occurred in 2 weeks, explants were kept for 6 weeks on rooting medium for further shoot growth and suberization of the cut surfaces.

2.4 Factors Affecting the Micropropagation and Root Induction System

Cytokinins. Despite the good results obtained with BA, the effects of other cytokinins were also screened. In the presence of BA no root formation occurs. This is also the case with zeatin mixed isomers (5–10 mg/l) and thidiazuron (1–2 mg/l).

On the other hand, root growth is visible on media supplemented with kinetin (2–4 mg/l) and 2iP (2–4 mg/l). The quality of the explants on media supplemented with zeatin can be compared to those with BA. TDZ causes a remarkable kind of tuberization without any shoot production. Concerning the influence of kinetin, it can be stated that roots are easily formed during the elongation of the leaves and shoots on MM. The problem is that it is uncertain whether the multiplication can always be sufficient if the second rooting medium is omitted. The effect of kinetin on the multiplication is different from that of BA, zeatin, or 2iP. With kinetin a few clearly defined shoots are produced. The numerous small axillary shoots or shoot initials that are commonly formed with the other cytokinins lack this compound. Such shoot initials have no real significance for the later development of the explants because they do not produce fully grown shoots. They were not considered for the multiplication ratio calculation.

Fig. 2A,B. Comparison of the development of roots formed in the presence **A** and absence **B** of NAA



The C-Source. Our experience with the use of carbohydrates in the culture of *Cordyline fruticosa* (Samyn 1995a), which is taxonomically related to the genus *Beaucarnea*, permitted some statements valid for both plants. It is logical that such important compounds in the medium, as carbohydrates will influence both the multiplication and the rooting phase.

Multiplication. Although a low sucrose concentration (1%) stimulates the formation of shoot initials in the in vitro formed tuberous aggregates, sucrose and glucose concentrations have no significant effect on the number of multiple shoot explants finally collected (Fig. 3). The multiplication ratio on media with sucrose and glucose concentrations between 1 and 4% is about 3 (unpubl. data). Explants to be transferred to the rooting medium are prepared with three well-developed shoots. Supplementary shoot initials do not develop on acclimatized young plants.

Root Induction. Figure 4 shows the influence of the concentration of sucrose and glucose during the second step on rooting medium. The number of roots is positively influenced by a higher (3–4%) sucrose concentration and reaches a maximum at 4%, although the root length does not change much between 2 and 4%. Although leaf development is not that much pronounced once the explant is placed on rooting medium, it is clear that higher carbohydrate concentrations, 3% glucose or 4% sucrose, provide an increased leaf length compared to the lower concentrations.

There is tendency to decrease the carbohydrate concentration in the medium during in vitro culture (Kozai and Kitaya 1993). The explants are pre-treated in this way to obtain a faster and a more efficient photosynthetic activity which will allow better acclimatization later on. The first findings during acclimatization of ponytail palm do not exclude the use of high carbohydrate concentrations. No difference was found between different amounts of the C-source of the medium.



Fig. 3. Difference in explant development caused by the carbohydrate concentration. *Left* 1% sucrose, *right* 4% sucrose

Fig. 4. **A** Effect of sucrose concentration in the rooting medium on the number of roots formed, the root development and the elongation of the leaves (means of 5 vessels with 10 explants each per concentration; vertical bars = ± 1 SE). **B** Effect of glucose concentration in the rooting medium on the number of roots formed, the root development and the growth of the leaves (means of 5 vessels with 10 explants each per concentration; vertical bars = ± 1 SE)

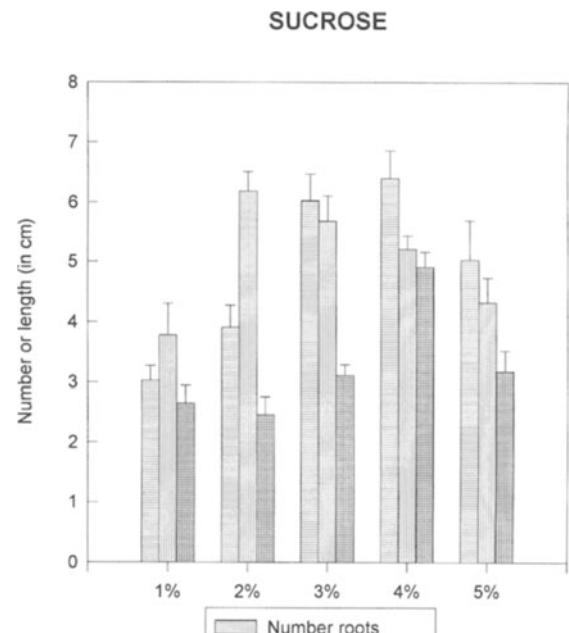
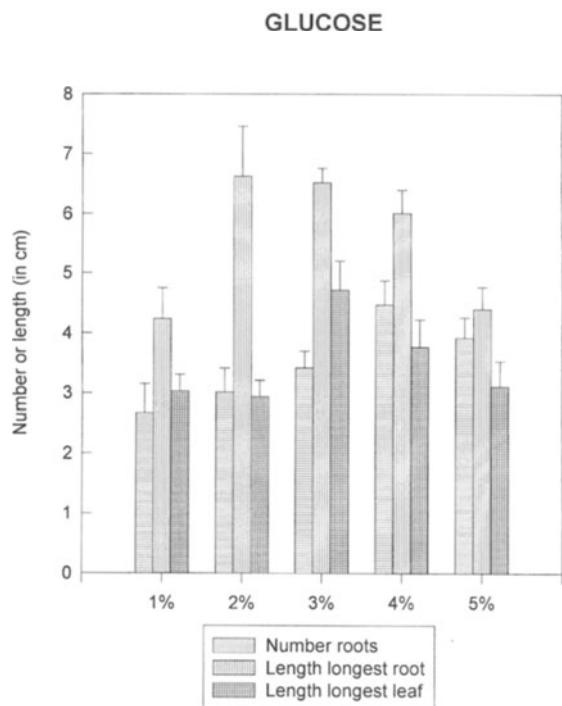
**A****B**



Fig. 5. Ponytail palm explants on BA ($2.2\mu\text{M}$) containing multiplication medium. *Right* after 2 months, *left* after 4 months, able to be divided and transferred to rooting medium

The more optimal in vitro growth obtained with high carbohydrate concentration should not be neglected. There are examples of other cultures where concentrations of carbohydrates up to 5% produce the best results (Samyn 1995b).

Length of Time That Explants Stay on the Multiplication Medium. The mean multiplication ratio of 3 obtained after a period of 2 months on multiplication medium containing BAP is sufficiently high, taking into account the fact that each obtained explant has several shoot apices. Most shoots, however, are not sufficiently elongated at that time to permit a problemless root induction, followed after 2 months by acclimatization. Elongation can occur in the vessel during the multiplication phase on MM after a further 2 to 3 months. It is assumed that the effect of BAP is lost after 3 to 4 months. The time spent on rooting medium should take no longer than 2 months, because leaf development is no longer important during this period and the quality of the roots declines once they have grown throughout the medium. After 4 months on MM the number of explants with sufficiently elongated leaves clearly increases (Fig. 5). The number of explants, after partition, which can be brought back to the multiplication medium proportionally decreases. A continuous in vitro system can be maintained for commercial purposes, by harvesting, as a function of the age of the culture, explants for a new multiplication subculture, or explants with elongated shoots that can be rooted (Fig. 6).

During the first 4 months, multiplication is essentially stimulated. After this the number of well developed and rootable explants increases. This means that this system requires several months. Ponytail palm culture has indeed no short culture cycles, and it should rather be compared to bromeliads or orchids, for which in vitro culture is nevertheless a most common technique for commercial practice. These remarks are only valuable for a system with BA.

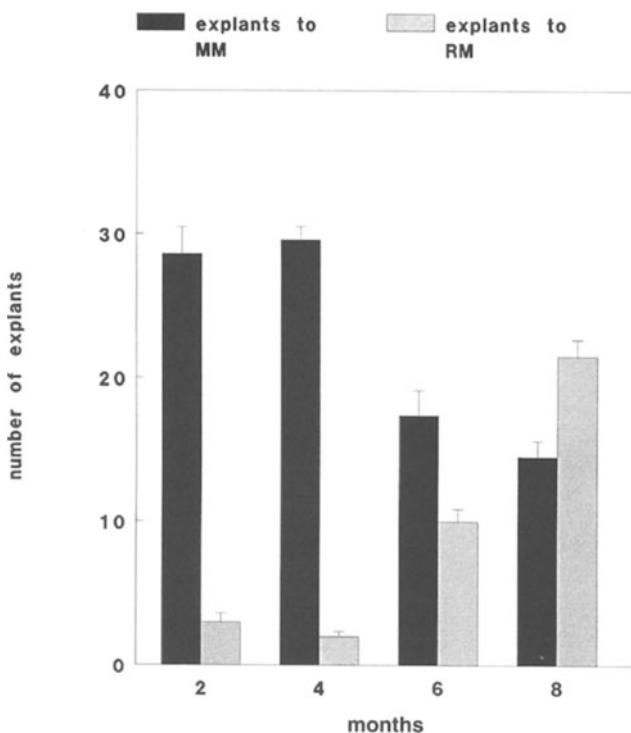


Fig. 6. Influence of the age of the multiplication cultures on the number of divided explants per vessel and on the relation between the number of explants able to be rooted and those to be subcultured again on multiplication medium (means of 20 containers with 10 explants each per time interval; vertical bars = ± 1 SE)

2.5 Acclimatization

Acclimatization occurs under normal greenhouse conditions at 20°C. It is not necessary to bring the plants into a 100% humidity environment just after transplanting, as is the case for most other in vitro cultured plants. Moist conditions are even harmful for the young tuberous stems, which can rot at the cut surfaces. After 4 to 6 months, plants are transplanted into 9-cm pots. They can be marketed in this pot after 1–1.5 years (Fig. 7). Growing larger plants with a trunk diameter of 20 cm or more takes several years. The care required by the plants is the same as for those obtained by seedling culture. One week after transplanting into a peat substrate, new root hairs are visible on the roots formed in vitro.

3 Schedule for Micropagation of Ponytail Palm

1. Initiation of apices, isolated from young selected seedlings: MS medium one-third strength without auxins.



Fig. 7. One year old "in vitro" propagated multiple shoot, a ponytail palm

2. Multiplication on Quoirin et al. (1977) medium supplemented with BA 0.5 mg/l. Duration of cycle: 3–5 months
 - alternatives: cytokinins: zeatin 10 mg/l
kinetin 2–4 mg/l
 - carbohydrates: glucose, sucrose 4%
3. Harvest the explants from multiplication medium and partition in explants with minimum three shoots before transfer to root induction and rooting medium.
Duration cycle: 2 months
MS salts one-third strength, supplemented with NAA 0.25 mg l⁻¹
sucrose concentration can increase up to 4%.
4. Acclimatization in normal glasshouse conditions without extreme humidity, but soon in full light intensity.

4 Summary

A successful in vitro culture of ponytail palm has to deal with two major objectives:

1. A sufficient multiplication ratio in order to obtain a commercially feasible production system, notwithstanding the presence of a certain number of shoots on one single explant. This permits the production of real multiple-shoot plants clearly different from seedlings which have only one apex.
2. A good root induction and root growth, which permits a fast and problemless acclimatization.

These objectives can be realized using two growth media, one with a high and the other with a low salt content, and two growth regulators. Multiplication is very well stimulated by BA. The auxin NAA, stimulating root induction, enables a fast and problemless acclimatization.

Axillary shoot stimulation must be compatible with further elongation and root growth. For this reason is it clear that the optimal cytokinin concentration will be lower than the maximum for multiplication. Considering the number of formed shoots, it seems that kinetin, at the concentration that root growth occurs, is inferior to BA. The effects of different C-source concentrations in a range of 1–5% do not offer really better perspectives compared to the direct effect of the cytokinin and auxin used.

The time during which explants can stay on the multiplication medium determines the production type. Fast multiplying cultures are easily obtained, but more time is required for the harvest of rootable explants.

A major advantage is the easy transfer from the glass vessel to glasshouse conditions, without any further precautions.

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I.20 Micropropagation of *Prunus tenella* (Dwarf Russian Almond)

L. BOUZA¹

1 Introduction

1.1 Botany, Distribution, and Importance

The dwarf Russian almond (*Prunus tenella*, $2n = 16$) belongs to the section Amygdalus of the family Rosaceae, and it is supposed to be a close parent of *P. communis*, the sweet almond (Krisa 1985). Two varieties of *P. tenella* exist: var. *Alba*, with white flowers and var. *Gessleriana*, with pink flowers (Chaudun 1982). In this second variety belongs the cv. *Firehill*, which has crimson-pink flowers and is the most important cultivar used as an ornamental (Fig. 1).

This plant originated in Siberia and northwest China (Sinkiang), but it is spread all around the northern hemisphere and especially in western Asia, southeast Europe, and North America (Krisa 1985). It grows on rocky, well-drained soils, in a large range of altitudes (0–1200 m; Yu 1979) and it prefers direct exposure to the sun (Hay and Beckett 1978). It is easily adapted to various climate conditions and it is very resistant to drought and cold (down to -30°C).

The dwarf almond is a compact shrub about 1 m high and 0.6–1 m wide, with very dense, upright slender branches of bright gray color, and it develops many suckers. Leaves are 3–7 cm long, dentate and sessile, of glossy green color, and they cover the whole branches (Chaudun 1982). Flowers blossom in March, before or at the same time as the leaves develop; they are borne on short shoots (brachyblasts), singly, or in groups of two to three (Krisa 1985). They are sessile and dense and cover the whole branches. Their five petals are irregular, 10–17 mm long and they have many stamens, which are shorter than the petals (Yu 1979). Fruits are not edible but they have an ornamental value. They are spherical drupes 1–2.5 cm long, of gray-yellow color, downy and rough, and are formed in June–July (Krisa 1985).

P. tenella is principally used as an ornamental shrub, and it received an Award of Merit in 1959 (Kelly 1986). Its compact shape makes it ideal for decoration of gardens or for the structure of narrow garden borders (Hay and Beckett 1978). The seeds are used for the production of a bitter oil, which is used in traditional Chinese medicine (Yu 1979).

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Fig. 1. Young plant of *P. tenella* cv. Firehill

Prunus tenella may also be a very useful source of genes for breeding of *Prunus* fruit crops (Yu 1979). Such fruit crops represent only a small fraction of the genetic diversity available within the species of *Prunus*. Breeding of these species could be done either by fusion of protoplasts or by the transfer of resistance genes (Scorza and Hammerschlag 1992), and an important breeding objective for them is a greater winter resistance (Bailey and Hough 1975; Hesse 1975) and, secondarily, drought tolerance. As *P. tenella* has these two qualities, it could be useful for such purposes.

1.2 Conventional Propagation and Need for Micropagation

The most common method for propagation of *P. tenella* is grafting (and especially T-budding) on seedlings of other *Prunus* spp. such as *P. avium* and *P. serrulata*, either in spring or in autumn (Kelly 1986; Hartmann et al. 1990). Grafted plants are saleable after 2.5–3 years and they are distributed to the market in pots. Despite the commercial importance of grafting some work has been carried out on propagation by cuttings. Semihardwood cuttings 12–15 cm long were used, and the only rooting treatment that allowed a satisfactory rooting (maximum 76% after 38 days) was a 5-s quick-dip in 1250 ppm K-NAA

+ 1250 ppm K-IBA aqueous solution; IBA alone was not effective (Kelly 1986). In addition, satisfactory but slow rooting of *P. tenella* cuttings was obtained by Nelson (1987) after dipping them in a 4% IBA rooting powder (70% rooting after 20 weeks); inoculation of these cuttings with an endomycorrhizal fungus (*Glomus intraradices*) did not improve these results.

Despite the work reported above, traditional propagation of *P. tenella* is still not financially attractive. Grafting has a maximum success of 80–90%, but is an expensive and time-consuming technique. Propagation by cuttings gave a maximum 76% of rooting, but cuttings were sometimes subject to decay and leaf drop (Kelly 1986). Thus, Alderson et al. (1987) estimated that a maximum success rate for propagation by cuttings was only 30%. For all these reasons, it would be interesting to replace traditional propagation by micropropagation. A large number of studies concern micropropagation of other *Prunus* spp. but, as far as we know, the only published work on micropropagation of *P. tenella* was done by Alderson et al. (1987). In their work, initial material came from shoot tips collected in June, and in vitro culture was carried out on MS medium (Murashige and Skoog 1962) supplemented with 1 mg l^{-1} (4.4 μM) benzylaminopurine (BAP). During multiplication, the most important problem faced was vitrification, and this was associated with a long sterilization before in vitro establishment (15 min). Vitreous explants died after being subcultured on fresh BAP medium. Non-vitreous explants gave the highest multiplication rate (MR) when 1.5 or 2.5 mg l^{-1} (6.6–11 μM) BAP were used, but vitrification and shoot-tip necroses were evident at these BAP concentrations. The addition of IBA to the medium reduced proliferation and caused shoot-tip necroses. In the same work, auxin was not found to be essential for rooting, as 25% of microcuttings rooted on a hormone-free medium. However, a maximum rooting of 42% was observed after a continuous culture on a MS medium in half strength containing 0.5 mg l^{-1} (2.5 μM) IBA. NAA gave the worst results as it caused callusing, and the roots were thick and brown.

2 Micropagation

2.1 Plant Material, Aseptic Preparation of Explants, and Subculturing

Initial plant material was axillary buds collected in spring from green branches of juvenile plants. Buds were surface sterilized in the following way: they were first soaked in a solution of Mercryl-laurilé (6%; Menarini, France) and shaken for 10–15 min; subsequently, they were soaked in an HgCl_2 solution (0.2%) and, after rinsing with sterile water (an important stage), they were dipped in a calcium hypochlorite solution (6%) and shaken for 20 min. Lastly, shoots were rinsed three successive times in sterile water. Initial explants developed axillary microshoots. These shoots were divided into four-nodal microcuttings and subcultured on fresh medium. For further multiplication the same process was followed; only high quality microcuttings were used, conse-

quently vitreous explants or non-elongated shoots (brachyblasts) were eliminated (Bouza et al. 1992). The multiplication rate (MR) was evaluated as the mean number of useful microcuttings obtained by one explant at the end of each multiplication cycle. Each culture cycle lasted 1 month and in total (from in vitro establishment until rooting process) 25 cycles were done.

2.2 Media and Culture Conditions

For in vitro establishment, a medium containing the salts of MS was used. For the multiplication phase, this mineral composition was compared with the same salts at half strength (MS/2) and with the salts of the Lepoivre (LP) medium (Quoirin et al. 1977). For rooting, the culture medium always contained the salts of the LP medium at half strength. All media were supplemented with the vitamins of Walkey in double strength (Quoirin 1974) and 3% sucrose. Hormones added to the multiplication medium were $2\mu\text{M}$ BAP, $0.6\mu\text{M}$ gibberellin A-3 (GA_3), and $1\mu\text{M}$ indole-3-butyric acid (IBA). Rooting occurred in two successive media: the root induction medium (RIM) contained IBA at different concentrations and the root development medium (RDM) was hormone-free. The pH of all media was adjusted to 5.5 and the media were solidified with 0.8% agar (Touzard and Matignon, France).

Explants (24 or 48 per experimental unit) were cultured in vitro in glass tubes of 20 mm diameter containing 10 ml culture medium. Two types of plastic tube caps were used to modify relative humidity inside the culture tubes (loose or tight ones). Room temperature was $25 \pm 1^\circ\text{C}$ and artificial lighting of $50 \pm 5\mu\text{E m}^{-2}\text{s}^{-1}$ was provided for 16 h day $^{-1}$ (Mazdafluor Prestilux-Incandia). For ex vitro establishment, vitroplants were transferred into pots with vermiculite and were watered regularly with a mineral solution of Nitsch (De Bilderling and Lourtiox 1976). During the first 10 days, plastic bags protected shoots from dehydration. Greenhouse temperature was 24 and 17°C for day and night, respectively, and a photoperiod of 16 h was maintained by natural lighting supplemented with artificial warm white lighting when needed, of a minimum energy of $125\mu\text{E m}^{-2}\text{s}^{-1}$ (Bouza et al. 1992).

2.3 Factors Affecting In Vitro Multiplication

Among the three mineral compositions tested (MS, MS/2 and LP), the LP medium gave the highest multiplication rate (MR) and the longer shoots (Table 1), without accentuating vitrification. The MS medium accentuated vitrification, and this can be attributed to its high NH_4^+ and Cl^- contents (Kevers et al. 1984; Gaspar et al. 1987). The MS/2 medium inhibited development and explants became chlorotic, a phenomenon already reported by Rugini and Verma (1983) when the MS/2 medium had been used for multiplication of *P. amygdalus*.

Another factor which affected both vitrification and explant development was the positioning of microcuttings on the culture medium. Table 2 shows

Table 1. Effect of the mineral composition of the multiplication medium on development of *P. tenella* explants. Data within a column followed by different superscripts are significantly different (Student's test, $P < 0.05$)

Mineral composition	Shoot length (mm)	Multiplication rate
MS	13.4 ^b	2.2
MS/2	8.6 ^c	0.8
LP	17.6 ^a	3.3

Table 2. Effect of the explant positioning on multiplication and vitrification of *Prunus tenella*. Data within a column followed by different superscripts are significantly different (Student's test, $P < 0.05$; Bouza et al. 1992)

Explant positioning	Multiplication rate	Percent vitrified explants
Vertical	1.4	0 ^a
Slanted 30° from horizontal	2.0	0 ^a
Horizontal	1.1	25 ^b

that the slanted explants gave higher MR than the horizontal and the vertical ones, and this position did not induce vitrification. The induction of vitrification in horizontal explants can be attributed to an excessive absorption of vitrifying elements from the culture medium (such as cytokinin, water, NH_4^+ and Cl^-), as previously suggested by Monteuijs (1986) for *Sequoiadendron giganteum* explants cultured in a horizontal position. The high MR of slanted explants could be due to suppression of acrotony and a more homogeneous development of axillary buds of these explants, compared to the vertical ones.

Relative humidity (RH) was found to influence MR and vitrification. Figure 2 shows that the intensive vitrification induced by a high RH (85%) of the culture chamber (RHch) disappeared immediately after transfer to lower RHch (45%), but the MR decreased concomitantly. To avoid this last effect, loose tube caps were replaced by tight ones; the consequent increase of the RH inside the culture vessel (RHv) restored the MR without increasing considerably the intensity of vitrification (Fig. 2).

It is generally admitted that a non-wounding stress factor (for example an excess of cytokinin) can induce vitrification (Debergh 1983; Kevers et al. 1984). To eliminate vitrification, BAP was omitted from the multiplication medium of *P. tenella*, for one passage. Although this treatment decreased the development of explants (only 37 instead of 96% of explants developed), vitrification completely disappeared. The subsequent transfer of explants to the standard multiplication medium restored development immediately (MR and shoot length), without inducing vitrification (data not shown). These results indicate that a reversion of vitrification can be achieved by a periodic omission of BAP from the culture medium, as already observed in *Betula* sp.

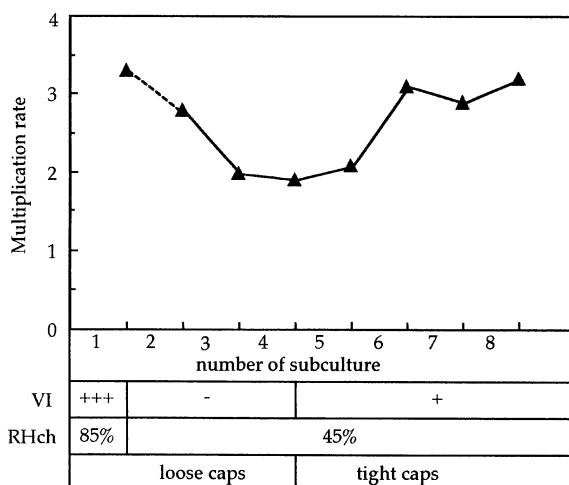


Fig. 2. Effect of the atmospheric humidity of the culture chamber and the tube-cap tightness on the multiplication rate and the vitrification of *P. tenella* cultured in vitro. Since the third subculture (first point of the *full line* curve), the selection of microcuttings subcultured was based on the elimination of all vitreous shoots. VI Vitrification intensity; – none; + low; +++ very high. RHch Relative atmospheric humidity in the culture chamber. (After Bouza et al. 1992)

by Böttcher et al. (1988). This reversion consisted in the development of non-vitreous shoots from vitreous explants and not the normalization of already vitreous tissues.

After several passages on the multiplication medium, the MR of *P. tenella* explants decreased progressively, due to a lack of shoot elongation. In fact, many axillary buds developed into brachyblasts instead of shoots (Fig. 3), consequently the number of microcuttings which could be taken at the end of the culture cycles was reduced (Bouza et al. 1992). It was supposed that an increase of gibberellin concentration could enhance shoot elongation, and consequently increase the mean number of microcuttings taken from one shoot (Druart et al. 1981). On the other hand, GA₃ has been found to decrease vitrification in *Dianthus* shoots (Böttcher et al. 1988), probably because of its effect on cell elongation. To test the effect of GA₃ on both shoot development and vitrification, we increased GA₃ concentration of the multiplication medium of *P. tenella* from 0.6 to 1.2 μ M. Results in Table 3 show that this treatment was not effective either in reducing vitrification or increasing the MR of *P. tenella* explants. This suggests that the progressive loss of shoot elongation capacity was not due to a gibberellin deficiency, but was probably dependent on other developmental factors.

It has been reported that the continuous in vitro culture of several ornamentals of the Rosaceae under stable temperatures caused a decrease in shoot length (Norton and Norton 1986), while micropagation of *P. cerasus* resulted in the early onset of dormancy in the young plants (Borkowska 1986). It is well established that the alternation of cold and warm conditions is an



Fig. 3. Explant during the 18th multiplication cycle: development of many brachyblast and only one shoot

Table 3. Effect of the GA_3 concentration of the multiplication medium on vitrification and shoot development of *P. tenella* explants. Data within a column followed by same superscripts are not significantly different (Student's test, $P < 0.05$)

GA_3 concentration (μM)	Percent vitrified explants	Shoot length (mm)	Multiplication rate
0.6	42	15.4 ^a	3.1
1.2	38	13.8 ^a	2.9

essential factor in releasing bud dormancy of perennials, including *Prunus* spp. (Dennis 1987), and cold was found to enhance shoot elongation of Douglas-fir in vivo (Pivert et al. 1986) and of *Paeonia* spp. in vitro and ex vitro (Albers and Kunneman 1992; Bouza et al. 1994). To test the effect of cold on development of *P. tenella* explants, a chilling treatment of 1 month at $+5^\circ C$ was applied. Results in Fig. 4 show that chilling remarkably enhanced both mean number of shoots/explant (while mean number of brachyblasts decreased) and mean shoot length. Consequently, the MR increased from 1.2 to 2.8 (Bouza et al. 1992). This result suggests either that continuous in vitro culture resulted in an explant degeneration, as suggested by Norton and Norton (1986), or that the

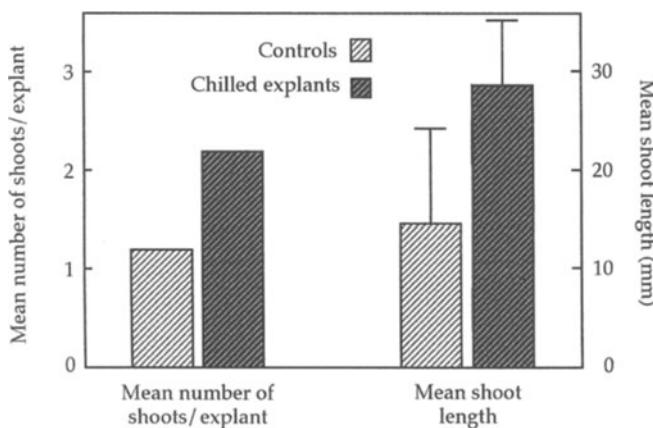


Fig. 4. Effect of a chilling treatment at +5°C for 1 month on the number of shoots/explant and the mean shoot length. Data were taken at the end of the culture cycle at standard multiplication conditions following the treatment. Bars = \pm SE

plant material conserved the need for seasonal fluctuations. We suppose that explants were induced progressively to a dormant state comparable to winter dormancy, but the addition of cytokinin and gibberellin in the culture medium allowed a minimal development. However, a chilling treatment was necessary to reactivate explants. Although it was recently shown that both GA and chilling of *P. persica* enhanced shoot elongation similarly (Frisby and Seeley 1993) and released similarly buds from dormancy (Luna et al. 1991), other studies showed that a GA treatment was less effective than chilling in releasing seed dormancy of *Pyrus* sp. (Bulard 1985) or on prolonging shoot extension of *P. cerasus* (Borkowska 1986). In this work, *P. tenella* presented a similar behaviour to these last two cases, as an increase in the GA₃ concentration did not enhance shoot expansion, whereas chilling did.

2.4 Factors Affecting In Vitro Rooting

To avoid the callusogenic effect of NAA reported by Alderson et al. (1987), in this work only IBA was used for rooting. Figure 5 shows that an auxin supply was necessary for root induction and that the optimum IBA concentration for root induction was 5 μ M, for a root induction period of 14 days. Roots developed after an induction either with 2.5 or with 5 μ M IBA were white, long and often ramified, while the ones formed after induction with 1 μ M IBA were short and did not ramify.

Root induction for a shorter period (7 instead of 14 days) was less effective, for the same concentrations of IBA used, while attempted root induction in a liquid medium containing 2.5 or 5 μ M IBA for 14 days was unsuccessful (data not shown).

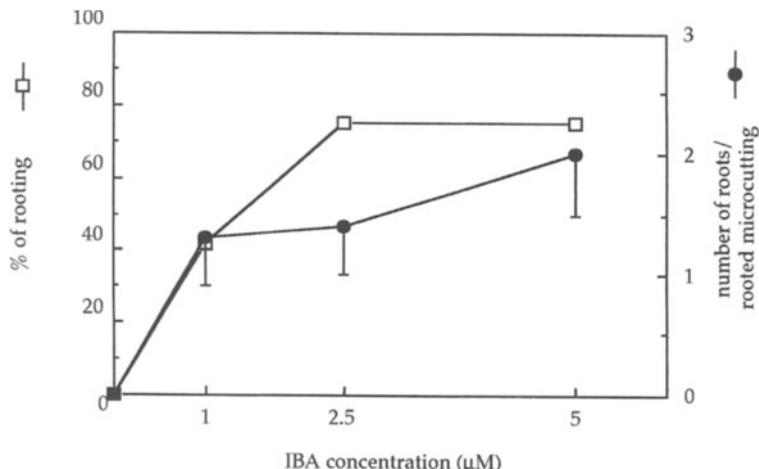


Fig. 5. Effect of the IBA concentration of the root induction medium (RIM) on rooting of *P. tenella* microcuttings. Results were taken after the sequence: 14 days on the RIM, followed by 30 days in the hormone-free root development medium. *Bars* = \pm SE

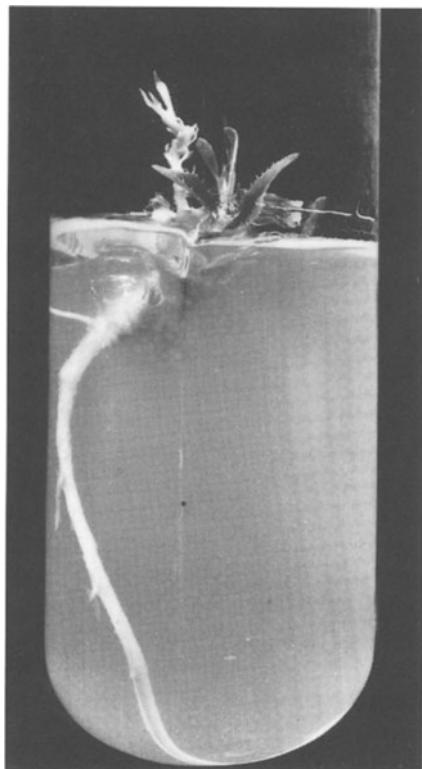


Fig. 6. Chlorotic vitroplant, induced for rooting on an IBA-supplemented RIM, in the dark

In many cases, root induction has been applied in the dark (Rugini and Verma 1983; Tricoli et al. 1985; Druart and Gruselle 1986), to avoid auxin destruction. In the case of *P. tenella*, although root induction in the dark allowed rooting, it caused defoliation and chlorosis to microcuttings both in the present work (Fig. 6) and in the work of Alderson et al. (1987).

In the above experiments, a total of 250 rooted vitroplants were obtained. The optimal conditions described gave 57 vitroplants with regular root systems and vigorous shoots.

2.5 Ex Vitro Establishment

After 30 days on the RDM, vitroplants were transferred ex vitro. All the 47 vitroplants coming from control multiplication conditions and a RIM containing 2.5 or 5 μ M IBA survived and developed vigorously (leaf size and shoot diameter doubled within 10 days). The ten vitroplants coming from an RIM containing 1 μ M IBA survived too, but started developing only 10 days after ex vitro transfer.

In addition, the chilling treatment applied during in vitro multiplication was found to be beneficial for ex vitro development also. In particular, two multiplication cycles after the chilling treatment described above (at +5°C for 1 month), microcuttings were subjected to the rooting treatment (on a RIM with 5 μ M IBA for 14 days and then on the RDM for 30 days). Six months after ex vitro transfer, shoots of chilled vitroplants were significantly higher than shoots of the controls; this was associated with both longer internodes and more internodes/shoot (Table 4). Vitroplants were transferred ex vitro in late spring. They developed continuously until the autumn, when they were exposed progressively to natural conditions. The following spring, all vitroplants flowered simultaneously and flowers were identical to those of the original cultivar. Thus, it seems that there is no risk of somaclonal variability of *P. tenella* after in vitro propagation by using axillary shoots. This confirms also that no juvenility was induced in the plant material during in vitro multiplication, which would delay flowering of vitroplants and consequently delay saleability.

Table 4. Effect of a chilling treatment during in vitro multiplication on ex vitro development of *P. tenella* vitroplants

Treatment	Mean number of internodes/shoot	Mean length of internodes (mm)	Mean length of shoots (cm)
Control vitroplants	15.9	72 ± 15	13.6 ± 5.3
Chilled vitroplants	25.4	95 ± 8	26.3 ± 9.0

Data ± SE were taken after the sequence: 1 month at +5°C; two cycles under the standard multiplication conditions; 14 days on the RIM; 30 days on the RDM; 6 months ex vitro (Bouza et al. 1992).

3 Summary and Conclusions

Factors affecting in vitro multiplication, rooting and ex vitro establishment of *P. tenella* were studied. Particular emphasis was given to vitrification in relation to proliferation and to the effect of chilling on shoot growth.

Vitrification occurs progressively during in vitro multiplication. This abnormality was controlled by using the salts of the Lepoivre medium, by culturing explants in a slanted position on the culture medium and by reducing the relative atmospheric humidity (RH) of the culture vessel. Moreover, it was completely eliminated by using a hormone-free medium for one passage or by radically reducing the RH of the culture chamber. However, factors eliminating vitrification reduced multiplication rate also. Thus, satisfactory proliferation was inevitably associated with the presence of some vitreous explants.

A progressive decrease in the length of explant shoots during in vitro multiplication resulted in a decrease in the multiplication rate. A gibberellin treatment did not improve shoot elongation while a chilling treatment was very effective.

Rooting was done in two successive media. The root induction medium always contained IBA, and root induction should not be done in the dark because it caused chlorosis and defoliation. Roots developed in a hormone-free medium.

Table 5. Protocol proposed for the micropropagation of *Prunus tenella* cv. "Firehill". RHch = relative humidity in the culture chamber

Culture step	Duration (days)	Mineral composition or substrate	Hormones	Environmental conditions	Other
Multiplication	30	Lepoivre	2 µM BAP; 0.6 µM GA ₃ ; 1 µM IBA	50 µE m ⁻² s ⁻¹ lighting, 16 h/day; Θ = 25 °C; RHch = 45%	Tight tube caps
Reactivation	30	–	–	+5 °C, dark	Only in case of elongation decline
Root induction	14	Lepoivre/2	5 µM IBA	50 µE m ⁻² s ⁻¹ lighting, 16 h/day; Θ = 25 °C;	–
Root development	30	Lepoivre/2	No hormone	Idem	–
Ex vitro establishment		– Nitsch mineral solution – Vermiculite	–	125 µE m ⁻² s ⁻¹ lighting, 16 h/day; Θ = 24/17 °C Day/night	Protection with plastic bags for 10 days

Ex vitro establishment was very successful for vitroplants having vigorous roots. Moreover, the chilling treatment applied during in vitro multiplication had a residual favorable effect on shoot elongation after ex vitro transfer.

4 Protocol

The micropagation protocol which allows the regeneration of thousands of vitroplants per year by one initial *Prunus tenella* explant is given in Table 5.

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I.21 Micropropagation of *Spiranthes sinensis* (Pers.) Ames (Orchidaceae)

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1 Introduction

Spiranthes sinensis (Pers.) Ames grows almost everywhere in open, sunny areas and even in disturbed areas, in eastern and southeastern Asia from Sakhalin and Siberia, Russia, to India and down to the eastern part of Australia. Since its natural variants such as variegated forms, double-flowered forms, and differently colored flower forms are horticulturally meritorious, they have been economically valuable and have often been cultivated.

Spiranthes sinensis has been micropropagated by us (Sato et al. 1987), and our technique, called “tissue-cultured shoot primordium method” has promise for clonal mass propagation, long-term conservation of the stable gene resource, and the artificial colchi-tetraploid of *S. sinensis*.

The tissue-cultured shoot primordium method was developed in 1983 for the purpose of long-term clonal preservation and micropropagation of the annual *Haplopappus gracilis* (Tanaka and Ikeda 1983). This technique patented in Canada, France, Germany and Japan can be applied to various plant species without any chromosomal aberration, genetic unbalance or virus contamination according to our experience (R. Tanaka et al. 1988; Kondo et al. 1991; A. Tanaka et al. 1991; Jin et al. 1993; Na and Kondo 1995). For instance, the tissue-cultured shoot primordia of *Haplopappus gracilis* have shown high abilities for regeneration of plantlets and rapid proliferation with chromosome and genetic stability during the course of more than 12 years culture.

2 Micropropagation Studies

The shoot primordium arises originally from an apical dome at the shoot apex, and can be maintained only in liquid media stirred in test tubes at 2 cycles per min by rotary culture equipment under 2000–10 000 lx illumination for 24 h daily (Tanaka and Ikeda 1983). The earliest tissue-cultured shoot primordium grows in culture largely up to the tissue called “secondary shoot primordium”

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and produces and multiplies another, new tissues called “primary shoot primordia” (R. Tanaka et al. 1988). The primary shoot primordium occurs in the outermost layer of a well-developed secondary shoot primordium or its surrounding inner region. Each primary shoot primordium consists of a few to 60 cells which are similar to each other in their round shape and their size (Tanaka and Ikeda 1983). After a few days it develops into a dome consisting of the secondary shoot primordium which is characterized by the outer single cell layer covering the inner cell tissue. After 3 to 4 weeks numerous shoot primordia united with each other and displayed a large, spherical mass 5–10 mm diameter which physically separates to form small pieces. Thus, spherical masses of shoot primordia propagate and occupy the 25 ml cubic content in a test tube 200 mm long × 30 mm diameter (Tanaka and Ikeda 1983).

2.1 Axenic Seed Germination and Protocorm Culture

Flowers of some diploid plants of *Spiranthes sinensis* collected in Hiroshima City were hand-pollinated. Ten- to 13-day-old, immature capsules were harvested, then surface sterilized in 1/5 Purelox (v/v) (sodium hypochlorite solution: 5% active chlorine) for 5 min, and finally rinsed a few times with sterile distilled water. Capsules were then dissected longitudinally and immature seeds were scooped out for sowing on solid medium (Sato et al. 1987). The Hyponex medium at a concentration of 2.5 g/l supplemented with 35 g/l sucrose, 2 g/l peptone and 0.9% agar (Kano 1965) was used for seed germination. Seeds were placed on 30 ml solid medium in 100-ml culture flasks and were incubated at 22°C under 500 lx illumination by a fluorescent lamp for 24 h daily. After 10 to 30 days, an average of 94.7% of the seeds germinated and began to form protocorms. This germination rate was better than that in *S. romanzoffiana* which germinated only under illumination, and that in *Spiranthes gracilis* which germinated only in the dark (Oliva and Arditti 1984). Additionally, seed germination of *S. gracilis* and *S. romanzoffiana* studied by Oliva and Arditti (1984) occurred on Curtis solution, that of *S. sinensis* studied by Nishimura (1982) occurred on Knudson C and Karasawa media, and that of *S. cernua* studied by Stoutamire (1964) occurred on a modified Knudson C medium but produced only white protocorms on the Norstog medium (Henrich et al. 1981). Symbiotic and asymbiotic germination and growth have been compared in *S. magnicamporum* by Anderson (1991) and *S. sinensis* var. *amoena* by Masuhara and Katsuya (1994). Thus the present, asymbiotic method of sowing the 13-day-old seeds on the Hyponex medium showed the best germination rate published so far.

2.2 Synthesis of Colchi-Tetraploid

Some diploid protocorms of *Spiranthes sinensis* in vitro were transplanted and cultured in the Hyponex liquid medium lacking agar and growth substances, but supplemented with colchicine at final concentrations of 0.0005, 0.005, and

0.05% in test tubes (30 mm \times 200 mm, 25 ml Hyponex liquid medium). The medium was stirred by rotary culture equipment at 2 cycles/min for 1, 2, 4, 6, and 8 days at 22°C under 2000–10000 lx illumination by a fluorescent lamp for 24 h daily. The protocorms were then rinsed with the Hyponex liquid medium a few times before they were transplanted to the Hyponex solid medium at 22°C under 4000 lx illumination by a fluorescent lamp for 16 h daily.

Only two of the protocorms treated with 0.05% colchicine for 2 and 4 days, respectively, produced perfect tetraploids, while the others were mixoploids (Table 1). The colchi-tetraploid and colchi-mixoploid protocorms were shrunken or irregular in shape. Thus, colchicine at concentrations of 0.005 and 0.05% induced C-mitosis of the protocorms, however, there were no anaphase or telophase stages after 2 days of treatment. Treatments of 10 days or more at these concentrations killed the protocorms. Colchicine at a concentration of 0.0005% did not alter cell division or chromosome number.

The colchi-tetraploid protocorms of *S. sinensis* obtained then began to differentiate to plantlets a month after transplantation onto the Hyponex solid medium.

2.3 Induction of Shoot Primordia

Perfect diploid and colchi-tetraploid protocorms of *Spiranthes sinensis* were placed in test tubes (30 \times 200 mm, 25 ml) containing B5 liquid medium (Gamborg et al. 1968) supplemented with α -naphthalene acetic acid (NAA) at

Table 1. Colchi-tetraploidization ($2n = 60$) in axenic-cultured protocorms of the diploid *Spiranthes sinensis* ($2n = 30$)

Colchicine concentration (%)	Treatment time (days)	Ploidy occurrence (cell no.)					Total
		2X	2X + 4X	4X	4X + 8X	Others	
0.0500	1						0
	2			1			1
	4			1			1
	6						0
	8						0
0.0050	1	1					1
	2	5	3	1		1 ^a	10
	4		2	2			4
	6	2	2	4	1		9
	8			1			1
0.0005	1						
	2						
	4						
	6						
	8		3				3

One protocorm was used for each trial.

^a $2n = 28 + 30$.

Table 2. Induction of tissue-cultured shoot primordia from shoot tips on both diploid and colchi-tetraploid protocorms of *Spiranthes sinensis* in B5 liquid medium supplemented and modified with NAA and BAP in combination at pH 5.7 for primary culture

NAA (mg/l)	BAP (mg/l)			
	0	0.02	0.2	2
0	Dead	Dead	PLBs calli SP or dead	SP or PLBs
0.02	Dead	SP PLBs dead	Calli dead	Dead
0.2	PLBs dead	SP PLBs dead	Calli MSs dead	MSs dead
2	Dead	PLBs dead	MSs dead	MSs dead

SP, shoot primordia; PLBs, protocorm-like bodies; MSs, multiple shoots.

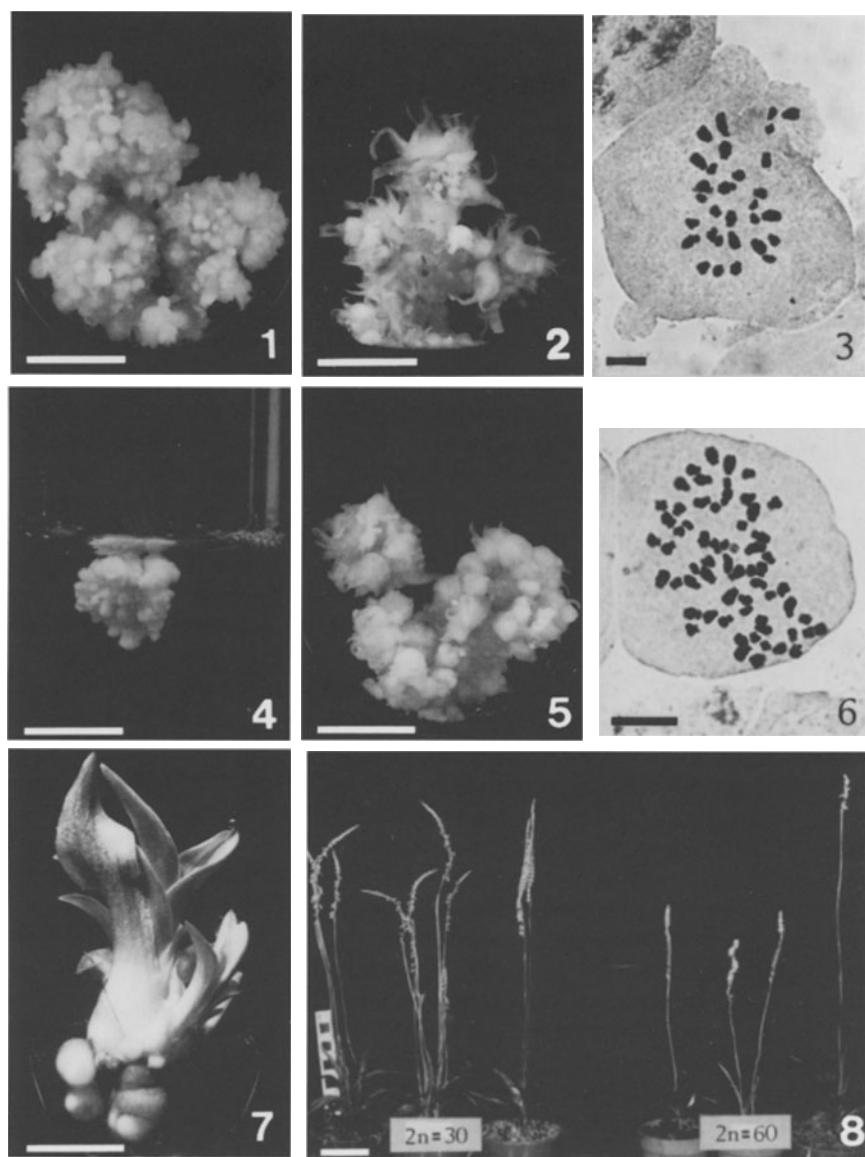
concentrations of 0, 0.02, 0.2, and 2 mg/l and 6-benzylaminopurine (BAP) at concentrations of 0, 0.02, 0.2, and 2 mg/l in combination at pH 5.7 for shoot primordium induction. The cultures were incubated at 22°C under 2000–10001x, illumination by a halogen lamp for 24 h daily and shaken at 2 cycles/min on rotary culture equipment (1-m diameter).

Four different cultures were obtained using different media during the course of primary culture. One culture, which was a bright-green mass 2 mm diameter with no shoot and no leaf, was isolated in B5 liquid medium supplemented with 2 mg/l BAP (Table 2). The mass had several domes and consisted of small cells in the epidermal layer and inner region large cells in the basal region. The small cells were filled with cytoplasm, while the large cells contained big vacuoles. The mass of small cells in the epidermal layer increased in size and developed gradually upward to organize a new dome. The histological characteristics of these tissues were typical of a mass of shoot primordia.

The shoot primordia were transferred and subcultured in B5 liquid medium supplemented with 2 mg/l BAP at pH 5.7 at intervals of 3 weeks.

2.4 Long-Term Storage of Shoot Primordia in Tissue Culture

Since the perfect diploid and colchi-tetraploid strains of the tissue-cultured shoot primordia were first developed and described in 1987, they have been conserved for 8 years in subculture, using B5 liquid medium supplemented with 2 mg/l BAP at pH 5.7. The masses of diploid shoot primordia are shown



Figs. 1–8. Cultures of *Spiranthes sinensis*. **1–3** and **7** Diploids. **4–6** Colchi-tetraploids. **1** and **4** Masses of tissue-cultured shoot primordia, *bar* = 1 cm. **2** and **5** Regeneration and formation of plantlets from tissue-cultured shoot primordia, *bar* = 1 cm. **3** Mitotic metaphase cell with the diploid chromosome number of $2n = 30$, *bar* = 10 μ m. **6** Mitotic metaphase cell with the colchi-tetraploid chromosome number of $2n = 60$, *bar* = 10 μ m. **7** Plantlet development, *bar* = 1 cm. **8** Flowering diploids ($2n = 30$) and colchi-tetraploids ($2n = 60$) regenerated from tissue-cultured shoot primordia, *bar* = 7.5 cm

in Fig. 1, and those of colchi-tetraploid shoot primordia in Fig. 4. They sometimes differentiated into multiple protocorm-like bodies (PLBs: diploid, Fig. 2; colchi-tetraploid, Fig. 5) in B5 liquid medium lacking any growth substance before regeneration of plantlets (Fig. 7) in the same medium. Both the diploid and colchi-tetraploid strains of shoot primordia have been well cloned and purified at intervals of 3 weeks during this 8-year course of conservation. Twenty-eight-day-old proliferation of the shoot primordia of the two chromosome strains was on average 7.14 times as much as the initial stage in every subculture. Thus, the proliferation rate of the shoot primordia was always much higher than that of the other cultures such as calli, multiple shoots, and PLBs (Sato et al. 1987). Stability in chromosome number of the subculture strains of the shoot primordia was examined periodically; the chromosome number of $2n = 30$ was always found in the diploid strain (Fig. 3) and that of $2n = 60$ in the colchi-tetraploid strain (Fig. 6) without any exception. Ability of regeneration of the shoot primordia to plantlets has been maintained and has shown an average of 1066 shoots/g weight of shoot primordia, which was much higher than that of the other cultures. The later development of plantlets into adult plants in both diploid and colchi-tetraploid strains was readily achieved (Fig. 8) without abnormality. Thus, the shoot-primordium method shows promise as a method of conserving plant gene resources on a long-term basis.

3 Summary

Spiranthes sinensis can be micropropagated and conserved for a long time by the “tissue-cultured shoot-primordium method.” The culture procedure was described as follows: numerous protocorms were successfully germinated from 10- to 13-day-old immature seeds on the Hyponex medium at concentration of 2.5 g/l supplemented with 35 g/l sucrose, 2 g/l peptone, and 0.9% agar at 22 °C under 500 lx full illumination. Only two protocorms treated with 0.05% colchicine induced perfect colchi-tetraploids. Then, both diploid and colchi-tetraploid protocorms synthesized shoot primordia in test tubes 30 mm in diameter and 200 mm long containing 25 ml of B5 liquid medium supplemented with 2 mg/l BAP at pH 5.7 at 22 °C on rotary culture equipment having a 1-m diameter shaking at 2 cycles/min under 2000–10000 lx full illumination. The shoot primordia were then transferred and subcultured in B5 liquid medium supplemented with 2 mg/l BAP at pH 5.7 at intervals of 3 weeks in the same environment. Both the diploid and colchi-tetraploid strains of shoot primordia were well cloned and purified during this 8-year course of conservation. They differentiated and multiplied PLBs and later regenerated plantlets in B5 liquid medium lacking a growth substance in the same physical condition when necessary during this 8-year course of conservation, culture, and investigation.

4 Protocol

The best explant material in *Spiranthes sinensis* was of protocorm; the best medium and culture conditions for microppropagation of tissue-cultured shoot primordia were B5 liquid medium supplemented with 2mg/l BAP at pH 5.7, rotary stirred at 2 cycles/min at 22°C under 2000–10000lx full-day illumination. Regeneration of plantlets was performed in growth-substance-free B5 liquid medium.

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I.22 Micropropagation of *Zinnia*

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1 General Account

1.1 Botanical Description, Distribution, and Importance

Herbaceous annuals in the genus *Zinnia* (family Asteraceae) are represented by a diverse array of classes based on flower heights, types, and colors. The popularity of *Zinnia elegans* Jacq. as a garden flower dates back to 1796, when it was introduced to Europe, and through decades of varietal selection it has become one of the top three choices for home gardeners (Mellon and Goldsmith 1985). While *Z. elegans* remains the most popular open-pollinated type in the genus, other species are gaining favor for certain landscape situations. *Z. angustifolia* HBK, which features excellent dwarf types, is currently soaring in popularity. The individual flowers of *Z. angustifolia* are quite petite and profuse, and this species is now widely used successfully in containers and as a bedding plant. An all-American selection of *Z. angustifolia* called Star White with a delicate, small white flower is typical of this species, called commonly the “classic zinnia” by growers and retailers. *Z. marylandica* (derived from a sterile hybrid of *Z. elegans* × *Z. angustifolia*, then restored in terms of fertility) is distinguished by superior disease resistance, as compared to *Z. elegans*. Hybrids are becoming increasingly popular, and most of the zinnias currently available are F₁ hybrids. These are currently the specialty of some large seed companies. *Zinnia* combines the advantages of visual variety, persistent bloom, excellent cut-flower keeping quality, and rugged tolerance to sun and water deficiency. Zinnias are also popular as pot plants (Fig. 1). They produce unisexual female ray florets and bisexual disk florets on the same flower head. Due to outstanding tolerance to summer heat, and drought tolerance, this crop has a broad distribution range, and is capable of performing in unfavorable landscape sites.

1.2 Conventional Propagation

Zinnia elegans is a seed-propagated crop, and production of hybrid seed for the gardener requires controlled cross-pollination of selected parental lines.

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Fig. 1. Zinnia flower. A wide variety of colors, heights, and habits characterize selections of this garden annual



Fig. 2. Individual flowers in a population from a single flower packet exhibit significant variability

The harvesting of very high quality seed requires rigorous insurance that only the intended parental lines are crossed. Lower quality seed sold as packets is often produced by interplanting a double inbred line (female) and a semi-double inbred line (male) with similar characteristics. However, since even a high degree of doubleness reduces, but does not completely prevent, pollen production, some mixtures of hybrid and inbred seed occur. Seed-propagated populations of a single hybrid line tend to exhibit distinctive variability between individual flower heads (Fig. 2). Inbred line development is further complicated by inbreeding depression caused by the accumulation of deleterious genes.

Commercial, high quality seed production from *Z. elegans* pivots on the exclusive use of a genic male sterility trait found in this crop. Vegetative propagation of identified female (male sterile) plants is considered too costly, therefore all major producers rely on male steriles and sib-crossing to maintain a line, which is later sown and rogued to eliminate plants that do not exhibit the male sterility trait (S. Pan, zinnia breeder, Pan American Seed, pers. comm.). Further details on the three-gene recessive male sterility trait and requirements for successful use are given below.

1.3 Why In Vitro Strategies Are Useful for *Zinnia*

Male Sterility as a Three-Gene Recessive Trait. Zinnia owes its broad range of selections to the consistent hybridization efforts of breeders. This hybridization can be facilitated by emasculation (physical removal of male flower parts from the female line), but it is a labor-intensive and cost-prohibitive procedure. Because of the composite flower head of zinnia, removal of fused male parts on an individual floret basis is not feasible.

Like many floral crops that capitalize on the uniformity and diversity of hybrid flower products, male sterile genotypes of zinnia provide a potentially valuable means of insuring 100% hybrid seed production. Male sterile flower lines permit controlled cross-pollination between the desired male and female lines, without uncontrolled and unwanted incidence of self-pollinations. Male sterility in zinnias is associated with an easy-to-mark apetalous trait (Fig. 3); therefore, through field roguing and rigorous progeny testing, the male steriles can be successfully used in commercial hybrid seed production (Metcalf and Sharma 1971). Because the genic male sterile trait in zinnia is governed by a three-gene recessive trait, its utilization typically demands stringent progeny testing to identify male fertile plants with a single dominant allele at any one of the three key loci. Clonal propagation of pure male sterile plant stands from identified specimens is prerequisite to the efficient use of genic male sterility in hybrid seed production (Rao et al. 1990).

Clonal micropropagation of selected male sterile female plants affords a convenient way of producing large uniform populations for cross-breeding, while eliminating labor-intensive roguing and progeny testing requirements. Greenhouse maintenance of a clonal female population produced by other means, such as cuttings, is subject to disease, insect infestation, and general decline problems which may invalidate the utility of the breeding line. Microculture, as opposed to any other clonal production method, facilitates production of large numbers of vigorous, disease-free parental plants in protected, aseptic maintenance conditions. Micropropagation, which is routinely used for these same reasons in clonal germplasm repositories, is the safest strategy for long-term maintenance of top quality parental stock.

Incidence of Unstable Alleles Conditioning Petal Pigmentation. Floral pigmentation patterns in some zinnia lines indicate that erratic gene expression conditions color expression. Ray flowers of these genotypes exhibit an irregu-



Fig. 3. The male sterile trait is characterized by apetalous flower heads

lar flecking pattern (Fig. 4), and flowers can be grouped into distinct genetic classes based on the relative activity of the unstable allele (Smith et al. 1988). Similar phenomena have been reported for other crops including maize, petunia, and Four O'Clock (*Mirabilis jalapa*). For zinnia, since the pigmentation pattern is located on relatively flat petals, it is easy to detect gene activity and locate specific cells in which the pigmentation gene is activated. An image analysis detection and quantification system was specifically developed for the zinnia crop to visually target distinct phenotypic classes, and was used to determine that excision does not appear to be under strict developmental control. A micropagation system for zinnia allows the multiplication and replication of distinct phenotypic classes, thus permitting further in-depth investigation of the genic instability, development of reliable interpretation methods, and biometric evaluation of the element's expression.

2 In Vitro Culture and Micropagation

2.1 *Zinnia* as an In Vitro Research Model

Table 1 summarizes in vitro studies involving the genus *Zinnia*. *Zinnia* has been the subject of in vitro research on tracheal development, xylogenesis, and

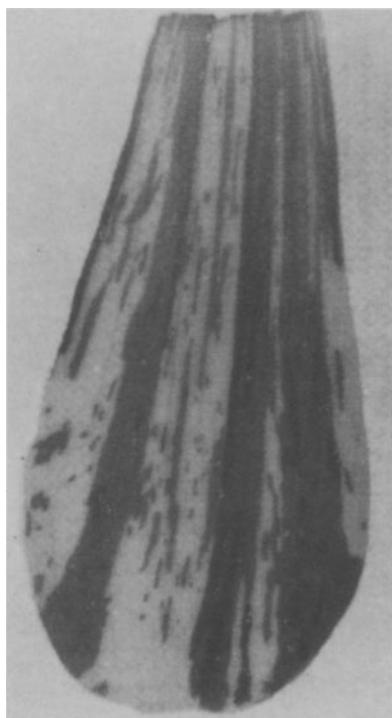


Fig. 4. Unstable allele expression in zinnia petals

Table 1. Summary of in vitro research, and micropropagation of *Zinnia*

<i>Zinnia</i> selection	Explant	Objectives	Reference
<i>Z. elegans</i>	Leaves and leaf cells	Protoplast isolation	Kohlenbach and Schopke (1981)
<i>Z. elegans</i>	Mesophyll cells	Tracheary element differentiation	Church (1993) Fukuda and Komamine (1983) Kohlenbach and Schopke (1981) Falconer and Seagull (1985)
<i>Z. elegans</i>	Mesophyll cells	Determine GA mode of action	Iwasaki et al. (1986)
<i>Z. elegans</i>	Axillary buds	Micropropagation of male steriles	Rogers et al. (1992)
<i>Z. marylandica</i>	Cotyledons	Somaclonal variation studies Adventitious shoot formation	Stieve and Stimart (1993) Stieve et al. (1992)
<i>Z. peruviana</i> × <i>Z. elegans</i>	Embryos	Embryo rescue	Shahin et al. (1971)

gibberellic acid activity (Kohlenbach and Schopke 1981; Falconer and Seagull 1985; Iwasaki et al. 1986). Embryo rescue of interspecific zinnia hybrids (*Zinnia peruviana* × *Z. elegans*) was also accomplished using in vitro techniques (Shahin et al. 1971).

Zinnia has served as a very attractive model system to examine aspects of differentiation and morphogenesis because the isolated cells or protoplasts,

for some reason, very readily exhibit spontaneous differentiation in vitro (Sugiyama and Komamine 1990; Church 1993). The study of cell differentiation in vivo is constrained by the lack of early markers to identify differentiation of cells (Thelen and Northcote 1989). Inducible in vitro systems are useful tools for dissecting the sequence of cellular and molecular changes, and for identifying regulatory mechanisms. Early research with zinnia cell cultures revealed a very high rate of tracheary element differentiation, which made zinnia an ideal model for morphogenesis research. Because single cells respond so predictably, problems with hormonal and nutritional gradients of multicellular organisms are avoided. Kohlenbach and Schmidt (1975) observed tracheal element differentiation in isolated *Z. elegans* leaf mesophyll cells in vitro in the presence of growth regulators, without an intervening cell cycle. The in vitro zinnia system was further refined by Fukuda and Komamine (1983, 1985), who achieved highly synchronous differentiation in the cell cultures. The zinnia model has afforded researchers an ideal model for the study of plant cell reprogramming (Sugiyama and Komamine 1990). It seems that zinnia has a unique ability in vitro to access alternative genetic programs from already differentiated states, which may be tied in with the wound response (Peter and Sussex 1994).

The zinnia system was successfully used to elucidate gibberellic acid action on cell division, again because a uniform single cell population could be induced to synchronous division, which is not the case with most other cell culture systems (Iwasaki et al. 1986). Other than the physiological/developmental research on *Z. elegans*, few other in vitro studies (for regeneration or production) have focused on this crop.

2.2 Somaclonal Variation in *Zinnia*

Somaclonal variation has been observed and studied extensively in adventitious shoots from *Z. marylandica* (Stieve et al. 1992; Stieve and Stimart 1993) as a means of exploring potentially useful variations from this selection. *Z. marylandica* is actually an amphidiploid created through a cross of *Z. angustifolia* and *Z. elegans*, and features enhanced disease resistance as compared to most currently available genotypes of zinnia for the home garden or cut-flower market. Traditional breeding for improvement was not feasible, due to sterility in the hybrid-derived selection. When adventitious shoots were regenerated from cotyledon tissues and grown on media with various TDZ levels, the somaclones exhibited substantial variation once grown to the flowering stage. Somaclonal variation was indicated in a diverse array of plant characteristics, including height, habit, petal color, and fertility. Significantly greater variation was produced in a population of somaclones as compared to a seed-derived population. Stieve et al. (1992) demonstrated a broad degree of phenotypic diversity in flower types, widely divergent in petal sizes, shapes, and spacing, in a line of progeny derived from the adventitious shoots. The researchers isolated several variants which exhibited strong, heritable varia-

tion (stably transmitted through sexual transmission), which have strong commercial potential. In particular, variants with novel petal colors and prolific seed set have been selected (see Stieve and Stimart 1996).

2.3 Micropagation

Micropagation tactics were developed for male sterile zinnias as a means to facilitate seed production, in-depth research into the male sterile trait, and other research focused on zinnias.

Physical environmental factors in the greenhouse where zinnia plants were produced (including light quality, photoperiod, and container size) not only had a significant influence on the general quality of the plants, but conditioned the abundance, size, and response of isolated explants once they were introduced to in vitro conditions. All phases of the micropagation procedure itself were investigated, from initiation of explants through proliferation, rhizogenesis, and acclimatization, and subsequent ex vitro performance of micropaginated male sterile plants.

Donor Plant Conditioning. A valuable clonal breeding line from zinnia, such as a genic male sterile line, must be carefully selected and isolated from other plants in a mixed population during roguing. In order to maintain these in vitro, it is particularly important to maximize the number of suitable explants that can be collected from individual, identified specimen plants. The growing conditions for greenhouse zinnia specimens used as explant donors prior to in vitro initiation had a significant bearing on the availability of suitable explants, on the incidence of explant contamination, and on the responsiveness of the explant to in vitro conditions. Photoperiod influence was investigated by imposing three separate treatment conditions on greenhouse plants propagated from commercial seed of the uniform, open-pollinated, partially inbred selection *Z. elegans* Orange Starlight: (1) natural daylight from 0700 to 1600 h, (2) photoperiod extension from 1600 to 2300 h (provided by 100-W incandescent lamps), or (3) daylight supplementation and extension from 0700 to 2300 h (provided by metalarc lamps). Evaluations of plant height, number of stem nodes, percentage of main stem axillary buds with >1 cm growth, and total number of buds and stem tips, were performed after 95% of the plants reached flowering stage (Table 2). Explants were collected from donor plants in each treatment at this time and evaluated for performance in vitro. Shoots produced from each set of explants were subcultured and reevaluated after three subculture cycles.

Donor plants from the latter treatment (with metalarc lamp photoperiod supplementation and extension) developed significantly more axillary branching than other treatments (Rogers et al. 1992). Photoperiod extension via metalarc lamps increased both the quantity and quality of explants. The length of time to flowering was reduced by over 3 weeks in the metalarc treatment, which made explant collection from this group far more efficient. Contamination was minimal for explants produced in the metalarc treatment, whereas

Table 2. The effect of light level and duration on the quantity of explant produced by *Zinnia elegans* Orange Starlight greenhouse stock plants. (Rogers et al. 1992)

Treatment	Plant height (cm)	No. of main stem nodes	Main stem axillary buds with over 1 cm of growth (%)	No. of visible buds and shoot tips per plant
Metalarc	23.4 c ^a	7.0 b	69 a	38.6 a
Incandescent	36.4 a	9.6 a	21 b	24.6 b
Shaded	31.9 b	10.0 a	7 c	13.1 c

^a Mean separation within columns by Student-Newman-Keuls test, 5% level.

Table 3. The effect of BAP concentration on axillary bud shoot proliferation of an identified male sterile zinnia selection. (Rogers et al. 1992)

BAP (μ M)	Degree of multiplication	No. of shoots larger than 2 cm	Shoot area (cm^2)	Callus diameter (cm)
0	3.2 b ^a	1.1 b	3.6 a	0.6 c
1	4.7 a	2.2 a	3.5 a	0.7 c
5	4.1 ab	1.7 ab	3.7 a	1.0 b
10	3.4 b	1.0 b	3.4 a	1.2 a

^a Mean separation within columns by Student-Newman-Keuls test, 5% level.

fungal and bacterial contaminations were a significant liability from the other donor plant conditioning treatments.

In separate experiments, growing the donor plants in a larger container size in the greenhouse (20- versus 15-cm plastic pots) influenced an increase in the length of branches arising from axillary buds located on the main stem, but did not significantly affect the number of available explants.

Proliferation from Axillary Bud Explants. Initial trials to screen for a suitable proliferation medium for zinnia also used *Z. elegans* Orange Starlight (Rogers et al. 1992). Seed was surface-disinfested after removal of the seed coats by immersion in 70% ethanol for 1 min followed by a 20-min soak in 1% sodium hypochlorite with 0.1% Tween-30 (Sigma Chemical Co., St. Louis, Missouri), then rinsed thoroughly in sterile deionized water. The germination medium consisted of half-strength MS salts (Murashige and Skoog 1962) without growth regulators. Shoot tips 1.5 cm long were collected from new growth generated in vitro, and 20 shoots were tested in proliferation media in each of the following treatments: (1) no growth regulators, (2) BAP (6-benzylaminopurine) at 1, 5, and 10 μ M concentrations with or without 0.5 μ M NAA (naphthaleneacetic acid), (3) kinetin or 2ip (isopentenyladenine) at 1, 5, 10, 15, and 20 μ M concentrations, and (4) TDZ (thidiazuron) at 0.01 and 0.05 μ M concentrations. After three consecutive subcultures at 2-week intervals, machine vision was used to measure shoot height, shoot area, callus

diameter, and degree of multiplication (Table 3). Shoot proliferation, good shoot quality, and maximal production of shoots over 2cm was achieved at low concentrations of BAP. Other growth regulator formulations resulted in excessive callus and adventitious shoot production (more prone to possible genetic variation), or root production at the expense of shoots (Rogers 1991).

For subsequent production of male sterile zinnia selections, a simple medium with $1\mu\text{M}$ BAP was used as the standard proliferation formulation (Rogers et al. 1992). Whereas at this low concentration of BAP, some problems with spontaneous root formation and excessive internode length were experienced with the Orange Starlight plants, these interfering events were completely suppressed in the male sterile plants. Axillary bud explants were collected from greenhouse plants during the flowering period so that the male sterile identity could first be confirmed. Male steriles produced in vitro very often exhibited premature flower bud formation, and the classic apetalous trait while in the culture microenvironment (Fig. 5). However, shoot cultures could be continuously maintained by carefully selecting two to three node stem segments and subculturing these explants on a 21-day cycle, to maintain vegetative growth.

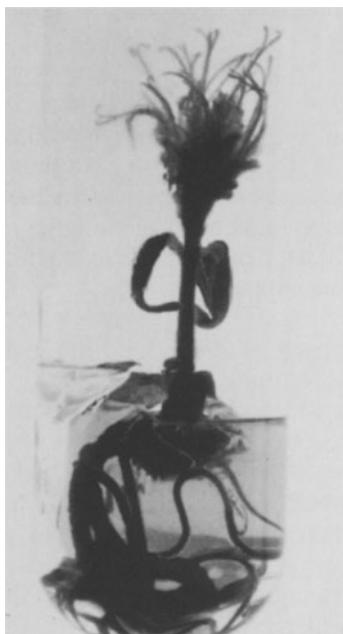


Fig. 5. An in vitro male sterile plantlet exhibiting early flower set. Vegetative shoot cultures of the male sterile plantlets could be continuously maintained on a medium with $1\mu\text{M}$ BAP. Excised male sterile microcuttings were easily rooted ex vitro, and acclimatized with a brief interlude under high humidity conditions

Microshoot Rooting, Acclimatization, and ex Vitro Performance. *Zinnia* microshoots as small as 2 cm rooted readily either in vitro (in a basal medium with no growth regulators) or ex vitro (in moist sterile silica sand). Cut ends of microcuttings were dusted with a commercial rooting powder (0.1% IBA, indolebutyric acid) prior to insertion in the sand, then held under high humidity fog conditions. The addition of any low concentration of auxin in the in vitro rooting medium led to excessive callus formation. Both in vitro and ex vitro rooting treatments successfully produced roots on 80% of microcuttings; however, the shoots continued to grow during the root initiation phase in vitro, and were therefore approximately 3 cm taller than ex vitro microcuttings at the end of the rooting period (10–14 days). Prior to acclimatization, rooted microcuttings were transplanted into 6.5 × 6.5 × 9 cm plastic pots in a potting soil. Acclimatization was easily accomplished in high humidity (fog) chambers for a few days, followed by direct transfer to a standard greenhouse bench, but attempts to acclimatize under mist resulted in microcutting loss to bacterial or fungal rot. Direct transfer from rooting stage to greenhouse bench (without an intermediate acclimatization under high humidity) resulted in smaller greenhouse plants, a condition that was maintained for the life span of the pot plants.

In order to evaluate the utility of micropropagated male sterile *Zinnia* plants for breeding, a micropropagated population was directly compared to seed-produced male steriles (selected after roguing). All plants were grown simultaneously under metalarc-supplemented lighting as described previously, and male fertile plants from the seed-sown population were discarded as identified. Each pollen parent was used to hand-pollinate equal numbers of in vitro-produced and seed-derived male sterile heads. Each head was pollinated for 3 consecutive days. The results were evaluated in terms of number of seeds set per head, number of branches, plant height, and head diameter (Table 4). Seed was collected and counted at maturity.

Although the height of in vitro produced male sterile plants was significantly less than for seed-derived counterparts, the degree of branching was enhanced, flower set was accelerated, and overall seed set was comparable to seed-derived plants (Table 5).

Table 4. Comparisons of in vitro and seed-derived male sterile *Zinnia* plants. Values are the means from 35 (seed) or 34 (in vitro) samples. All measured traits were significantly different between in vitro and seed-derived plants at the 0.01 probability level (Rogers et al. 1992). SPH = seeds per head.

Traits	In Vitro	Seed
Height (cm)	25.60	36.08
Number of branches	8.18	5.80
Seed head diameter (cm)	1.98	2.24
SPH	17.21	28.07

Table 5. Correlations and significance levels among traits for seed-derived (N, 35) and in vitro-derived (N, 34) male sterile plants. (Rogers et al. 1992)

	Number of branches	Diameter	Seeds per head
Seed-derived male steriles			
Height	0.43 ^a 0.011 ^b	0.52 0.001	0.62 0.0001
Number of branches	–	0.09 0.62	0.48 0.003
Diameter	–	–	0.55 0.0006
In vitro produced male steriles			
Height	–0.20 0.25	0.53 0.001	0.07 0.71
Number of branches	–	–0.31 0.08	0.12 0.49
Diameter	–	–	–0.07 0.69

^aCorrelation coefficient.

^bSignificance level.



Fig. 6. The diversity of flower types, colors, sizes, and other ornamental characteristics in the *Zinnia* genus have increased sales in the United States and worldwide

3 Commercial Aspects

Several major nurseries and seed companies are mass-producers of *Zinnia*; both species and hybrid selections. The striking diversity of floral types and colors featured in trial display gardens and nurseries (Fig. 6) have increased public interest and resulted in a larger volume of production through commercial growers (Y. Arimitsu, zinnia breeder, Bodger Seed Co., pers. comm.). Major producers such as Goldsmith Seed, Takii Seed, and Pan American Seed

have responded to the expanding market by specializing in breeding and mass production of hybrid selections. Other large growers, such as Bodger Seed Co., concentrate on the open-pollinated selections, primarily of *Z. elegans*. While all large scale commercial propagation is almost exclusively done by seed propagation (S. Pan, zinnia breeder, Pan American Seed, pers. comm.; Y. Arimitsu, zinnia breeder, Bodger Seed, pers. comm.), vegetative multiplication of selected parents is desired for hybrid breeding. Vegetative propagation in particular would alleviate heavy breeder reliance on roguing to maintain pure lines. *Zinnia* does not respond well to cutting propagation, primarily because of poor rooting response and extreme susceptibility to propagation disease. This limitation to bulk-up of valuable breeding germplasm is alleviated through in vitro propagation, since microplants root readily and are safeguarded from fungal or bacterial pathogens during multiplication.

4 Summary and Conclusions

Zinnia genotypes, in particular *Z. elegans*, have long provided an excellent model for a series of in-depth physiological investigations in vitro, as noted previously. In light of the popularity of the crop, and the strong need for vegetative methods to maintain some complex genetic lines (such as the male steriles) for breeding, it is somewhat remarkable that zinnia micropagation research, with direct significance for the production industry, has only recently been reported. Within the past 5 years, micropagation protocols, adventitious shoot regeneration techniques, and potentially useful somaclonal variation have all been demonstrated successfully for zinnia. In vitro techniques clearly provide a reliable way to produce and maintain valuable parental lines without the liabilities (disease and declining vigor) typically associated with long-term maintenance under greenhouse conditions. The short time between subcultures (~2 weeks) and the high degree of multiplication would allow the potential production of over 1000 rooted plantlets per initial explant in just over 3 months (Rogers 1991). With this rate of clonal propagation, a single donor stock plant grown under the optimal environment (metalarc lamp-supplemented) could potentially generate over 39 000 rooted plantlets within the 3-month interval. Given the small size of seed plots for a given hybrid (4–5 acres, J. Gibson, Goldsmith Seeds Inc. Gilroy, California, pers. comm.), a commercial grower could feasibly generate the number of male sterile plants required for the female line within a short time frame. In vitro-derived seed parents have a comparably high seed yield, and as an added bonus, are capable of setting flower buds earlier than seed-derived plants.

5 Protocol

Axillary bud explants are collected during the flowering stage from donor greenhouse plants grown with supplemental metalarc illumination. Explants are surface disinfested by a 15-s immer-

sion in 70% ethanol, followed by agitation for 15 min in a 1% sodium hypochlorite solution, and thorough rinsing in sterile double distilled water. Explants are trimmed of damaged tissue and placed in a basal medium with 1 μ M BAP. Microcultures exhibit limited shoot proliferation and occasional spontaneous rooting under these conditions. Microshoots excised at the medium surface interface and placed vertically in a growth regulator-free medium for rooting, respond with at least 80% rooting, and each of the rooted shoots can easily be acclimatized to ex vitro conditions in a lighted growth chamber with fog. Once placed in a typical greenhouse pot plant environment, the micropropagated male sterile zinnias rapidly set floral buds, and can be used in cross-pollination trials as a uniform female parental line.

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Section II

Medicinal, and Miscellaneous Plants

II.1 Micropagation of *Duboisia* Species

O. LUANRATANA¹

1 General Account

1.1 Botany, Distribution, and Importance

The genus *Duboisia* (family Solanaceae), containing only three species, is native to Australia. Each species is located in a distinct area: *Duboisia hopwoodii* F. Muell. in arid Central and Western Australia, *D. leichhardtii* F. Muell. in a very restricted area of southeast Queensland in semi-evergreen vine thickets (dry vine forests), and *D. myoporoides* R.Br. along the eastern coast from the south of Sydney to beyond Cairns in North Queensland is found in notophyll vine forests (Barnard 1952; Webb 1959; Griffin 1985; Pole 1993).

D. myoporoides is a tree with broad-lanceolate to obovate glabrous leaves 7.5 to 10 cm long which may grow 13 m tall. The flowers are small, white, and bell-shaped with occasional mauve streakings in the throat of the corolla. The fruit is a small black globular berry about 0.5 cm in diameter. *D. leichhardtii* is smaller than *D. myoporoides*, with narrow, lanceolate leaves and a little larger flower. *D. hopwoodii* is a small shrub, seldom exceeding 2.5 m in height, with glabrous and very narrow lanceolate leaves which are smaller than those of *D. leichhardtii*, and small bell-shaped flowers (Bottomley and White 1951).

D. myoporoides was first discovered in Australia by Robert Brown in the Flinders expedition of 1802–1805. The second species, discovered in 1872, was *D. hopwoodii*, and the last, *D. leichhardtii*, was described in 1877.

The medicinal value of these plants was recognized in 1861 during the Burke and Wills expedition. One of the survivors had an experience with the narcotic “Pituri” given to him by Aborigines. He reported that chewing Pituri made him forget his hunger and miseries. The plants used for the preparation of Pituri were *Nicotiana excelsior*, *N. gossei* and *D. hopwoodii*. Several samples of *D. hopwoodii* from Central, South and Western Australia were collected and analyzed for their chemical composition. Nicotine was the major alkaloid in the Western samples, however, the samples from Central and South Australia contained only nornicotine (Coulson 1967). Later work has shown that

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Pituri was prepared from Western *D. hopwoodii* whose major alkaloid was nicotine. The other type of *D. hopwoodii* from Central Australia contained nornicotine as the major alkaloid and was used to poison emus (Bottomley and White 1951; Luanratana 1982; Watson et al. 1983).

The aqueous extract of *D. myoporoides* injected into cats and dogs showed different pharmacological effects from those produced by Pituri extracts. Instead of excitation and vomiting followed by respiratory paralysis, the animals appeared to be almost blind, became sleepy, and the pupils were widely dilated. Mydriatic effects were observed when the extract was dropped directly into the eyes of both animals and humans. By 1887, it was clear that some leaf samples of *D. myoporoides* contained hyoscine, some hyoscyamine, and some both alkaloids (Coulson 1967). Later in 1945, two types of *D. myoporoides* were identified from the north and south of Gosford, New South Wales. The former contained hyoscine as the main alkaloid, while the latter produced mainly hyoscyamine. The types were designated as the Northern and the Southern varieties, respectively (Coulson 1967).

Another type of *D. myoporoides* was reported in 1953 when seeds of New Caledonian origin were grown to maturity in Canberra. Nornicotine and nicotine were isolated in addition to hyoscine and hyoscyamine. This was the first time the tropane alkaloids and pyridine alkaloids were reported in the same plant. Four years later a sample leaf of *D. myoporoides* from the Acacia Plateau on the Queensland-New South Wales border was analyzed and revealed the presence of hyoscine, nicotine, anabasine, and isopelletierine. The alkaloids of the aerial and root parts were studied extensively by Coulson and Griffin (1967, 1968).

The leaves of *D. leichhardtii* contain hyoscyamine, hyoscine, valtropine, butropine, and norhyoscyamine (Griffin 1965).

1.2 Conventional Propagation and Cultivation

Commercial exploitation of *Duboisia* as a source of hyoscine and hyoscyamine commenced in 1940 (Coulson 1967). Formerly, *Duboisia* leaves were collected from natural stands until after World War II, when the need for cultivation of high alkaloid yielding plants was recognized (Kelenyi 1949).

Farmers in Australia grow a hybrid which is believed to be derived from the work of Loftus Hills et al. (1954) and is probably the progeny from a male *D. leichhardtii* and a female parent of the Northern variety of *D. myoporoides*. The hybrid shows advantages over the parent species: more vigorous growth, higher hyoscine content, and less complex alkaloid spectrum. The pyridine alkaloids which are present in *D. myoporoides* are absent from the hybrid (Griffin 1985). Cuttings are usually prepared from the lower branches (D. Etherington, a *Duboisia* farmer in Proston, Queensland, Australia, pers. comm.) and dipped into root-promoting powder containing indolebutyric acid (IBA) as the active constituent. The cuttings are then kept in a mist box in the shade for 1–2 months and hardened under field conditions for 4–8 weeks before planting.

Experiments in Thailand were carried out on 400 cuttings of an imported *Duboisia* hybrid in the dry hot season (March). Only 40 cuttings developed roots but died in the process of hardening off. Later in the cool season (October) 116 rooted cuttings were obtained from 180 imported cuttings after being kept in a mist box for 6 weeks. After transferring to a pot mixture the number was reduced to 80 due to damage to the root system during transfer. Only 38 rooted cuttings survived the hardening process and were planted in the field in Chantaburi (Luanratana 1989a).

Duboisia species are fast-growing trees and give higher yields of tropane alkaloids than other solanaceous plants. Repeated harvests can be obtained every 3 months in a tropical climate, whereas in a cool climate repetition can be done at 1-year intervals (Loftus Hills et al. 1954). A few countries have developed plantations of *Duboisia* spp. Cultivation studies of *D. myoporoides* in Japan commenced in 1961. The plants were nurtured from imported seeds. The total alkaloid yield was higher in summer than in winter, 1.53 and 1.13%, respectively. The hyoscine content was 22–25% of the total alkaloids and the content of hyoscyamine was half of that of hyoscine (Miyazaki et al. 1977). Factors affecting alkaloid yield were also studied extensively, including soil water and seasonal effects. It was noted that soil water had effects on height and primary branch number; 60–80% soil water was suitable for leaf growth and hyoscine content, but the total alkaloid yield was highest at 40% soil water; plant growth was severely reduced at 20% soil water. Maximum water absorption from roots was highest during 12–16 h during a day when the temperature reached its highest and the humidity was lowest (Ikenaga et al. 1977a,b). The plants showed better growth and higher yield of total alkaloid and a higher hyoscine content in warmer months. Location also affected growth of the plant and a comparative study was made between cultivation in Okinawa which lies at a latitude equivalent to that of Proston in Australia, and Nagasaki where the climate is colder. As might be expected, the plants grew better in Okinawa, and harvesting of leaves could be repeated in 9 months (Ikenaga et al. 1978).

The cultivation of *D. myoporoides* was also tried in India in 1979 with the intention of exploiting the plant commercially as a source of hyoscine. The plants were grown from seeds and could be harvested at the age of 10 months, yielding different quantities of hyoscine and hyoscyamine. Different plant spacings did not affect the leaf yield significantly (Singh et al. 1985).

D. leichhardtii was cultivated in western Java, Indonesia, as the source plant for hyoscyamine for a Japanese pharmaceutical company (Eisai Co. Ltd., Kawashima, Hashima, Gifu). The plants contained 1.59% hyoscyamine and 0.64% hyoscine (Kagei et al. 1978).

Experimental cultivation in Thailand has been carried out since 1989. The cultivation was tried in three different locations. Firstly, the regenerated plants of *D. myoporoides* and cutting-derived plants of *Duboisia* hybrid introduced from Australia were grown on volcanic soil in the Chantaburi province (Kityaruk et al. 1985), eastern Thailand. The plants grew vigorously reaching 3 m in height in the first year. Pruning every 3 months and supporting of the

main stem were necessary, otherwise the plants gradually lost their vigor and died (Luanratana 1988, 1989a,b).

Analysis of leaf samples collected from over ten plants during October 1989 to April 1990 showed quite stable hyoscine content with hyoscyamine as the second major alkaloid. The field-grown hybrid plants were more robust than other species, and showed higher survival rates. Hyoscine was also the major alkaloid in the hybrid with hyoscyamine as the second major alkaloid (Fig. 1). There were no sharp decreases in hyoscine content as reported in Japan and Australia; this could be due to the narrower temperature range, 18–39°C (Luanratana 1990a).

In our present studies, two experimental plots were set up in Nakorn Pathom approximately 60km from Bangkok in 1992 and 2 years later in Nakorn Rachasima, northeastern Thailand. The plants were all derived from tissue culture. One hundred regenerated plants of *D. myoporoidea* were gradually transferred to each plot. Both locations were found to be more suitable than Chantaburi and a few 2-year old plants in Nakorn Pathom yielded flowers in June 1994. The flowers are small, white, and bell-shaped with some mauve streakings in the throat of the corolla (Fig. 2I).

The field plants of *D. myoporoidea* in Nakorn Pathom were more robust than those grown in Chantaburi (Fig. 2G). However, analysis of the leaf samples collected individually from 32 plants in January 1994 revealed an average of 0.72% hyoscine and 0.13% hyoscyamine g dry weight. More than half of the plants gave a greater hyoscine yield than 0.7%. The highest hyoscine content measured was 1.17% and the highest hyoscyamine content was 0.18%. Although the percentage yield of alkaloids was lower, a higher leaf yield resulted in higher total yield of alkaloid per rai (ca. 41 acres) (Luanratana 1994). In addition, the field plants also showed a higher survival rate (88%). Analysis of leaf samples taken monthly from four groups of plants classified according to their ages during January–September 1994 revealed a small reduction in hyoscine content in May when rain was abundant (Fig. 3). The effect was more pronounced with bigger plants which suffered from restricted drainage due to soil type (Luanratana 1995).

Harvesting by cutting the main stem down by one third could be done every 3 months, confirming a previous report (Loftus Hills et al. 1954); on average, each plant would give a dried leaf yield of as much as 2 kg/year. The plants were spaced at 1 m × 1 m intervals, therefore one rai (ca. 41 acres or 1600 m²) would accommodate 400 plants and the yield per rai would be 800 kg of dried leaf.

The plants grown in northeastern Thailand showed more vigorous growth than plants in central and eastern Thailand. The field plants could reach the height of 1.8 m in 6 months (Luanratana 1994). Examination of leaf samples collected individually from 29 plants (10 months old) revealed higher hyoscine and hyoscyamine contents. The highest hyoscine content measured was 1.19% and highest hyoscyamine content was 0.29% (Luanratana 1995). Recently, four regenerated plants of *Duboisia* hybrid were grown successfully under

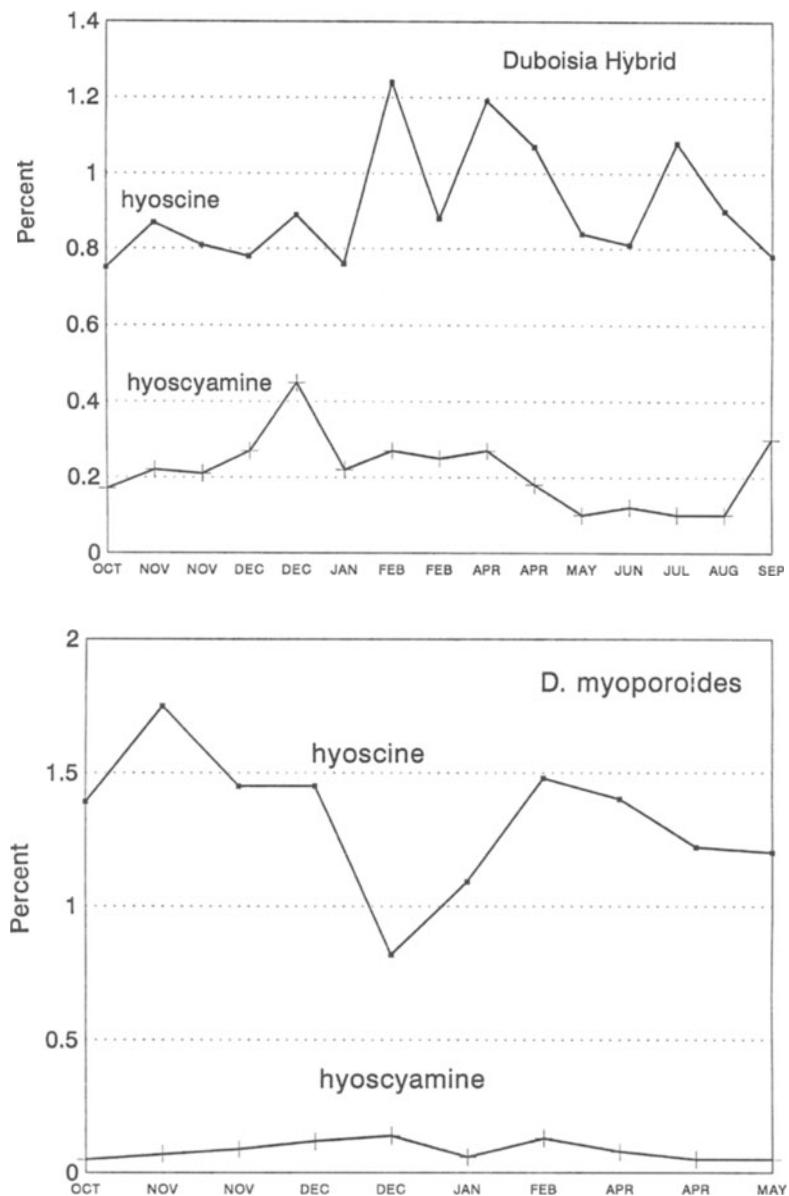


Fig. 1. Variation in alkaloid content in field grown *Duboisia* spp. during October 1989–May 1990, Chantaburi. (Data from Luanratana 1990b, 1991)

field conditions at Nakorn Pathom. After 4 months the plants reached 25 cm in height and one of the plants showed vigorous growth. Analysis of the leaf sample revealed 1.49% hyoscine and 1.46% hyoscyamine (Fig. 4; Luanratana 1995).

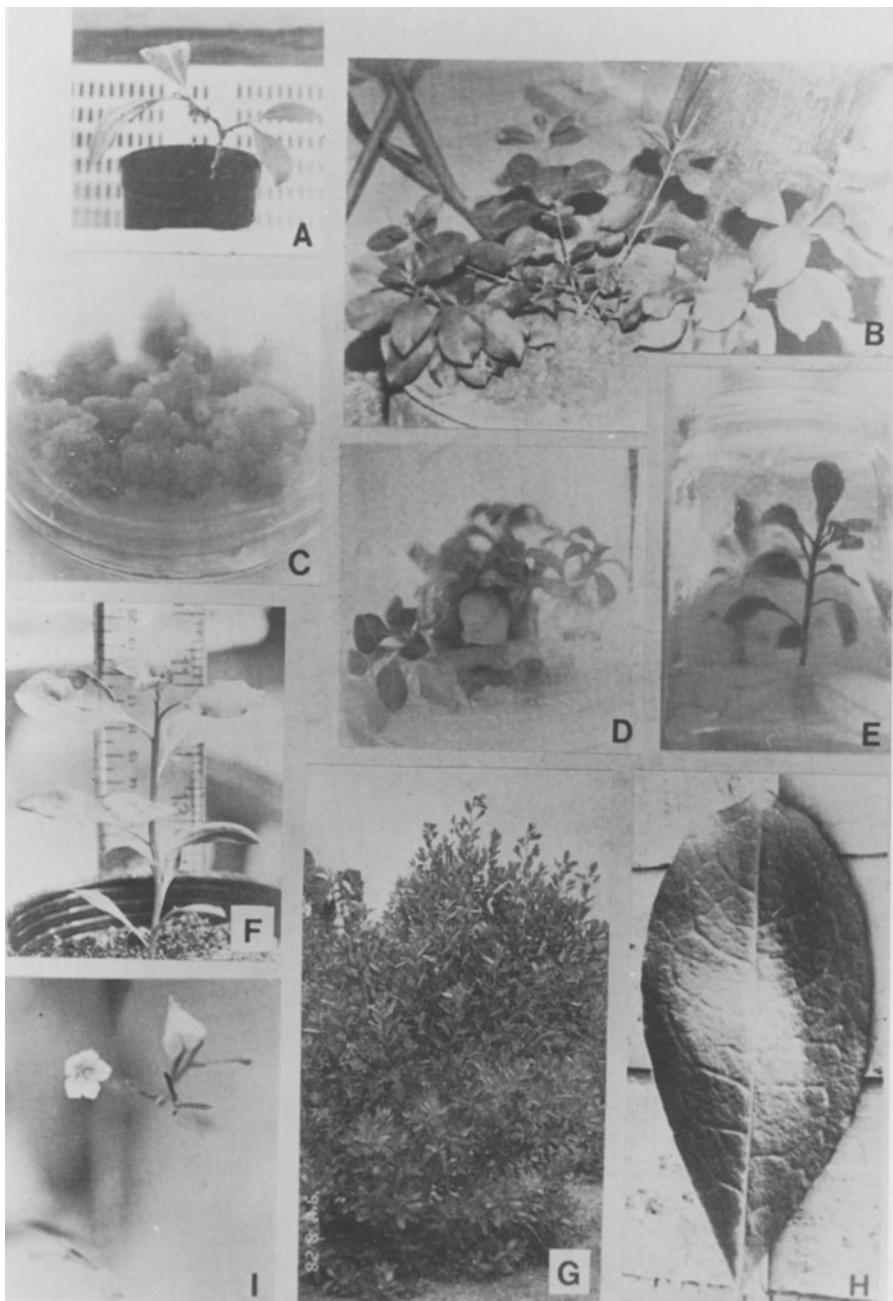


Fig. 2. Micropropagation of *D. myoporoides*. **A** An imported cutting with roots, Bangkok 1988. **B** The mother plant at 6-months in pot, Bangkok 1989. **C** Callus subcultured for 6 years on RT medium containing 2,4-D (1 mg/l), IAA (1 mg/l), and BAP (0.6 mg/l) under light, 3 weeks after transferring to fresh medium, 1994. **D** Multiple shoot development from callus, cultured on RT medium containing NAA (4 mg/l) and BAP (4 mg/l) for 4 weeks, 1994. **E** The regenerated plants derived from shoots cultured on RT medium containing IBA (4 mg/l), 1994. **F** Two weeks after transplantation, 1994. **G** The regenerated plant after 6 months growth in the field (Nakorn Pathom), 1994. **H** The mature leaf from field-grown plant. **I** The flower, Nakorn Pathom 1994

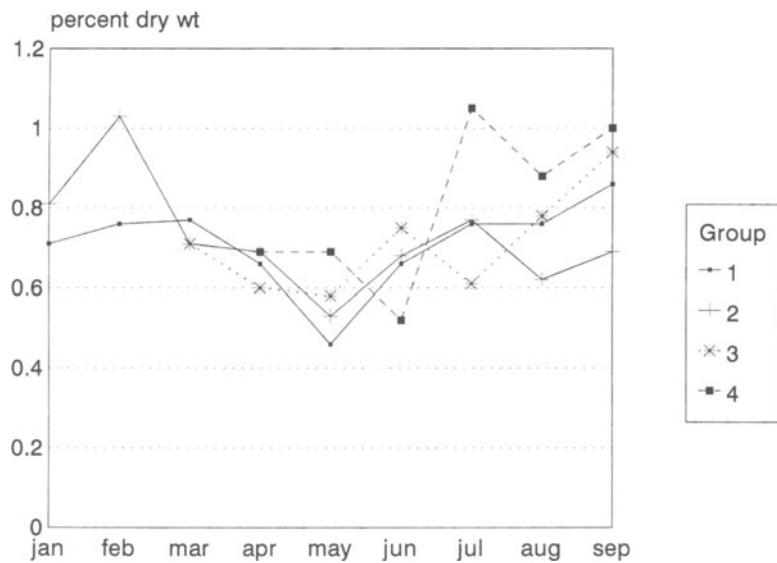


Fig. 3. Variation in hyoscine content in *D. myoporoides*, Nakorn Pathom. (Luanratana 1995)

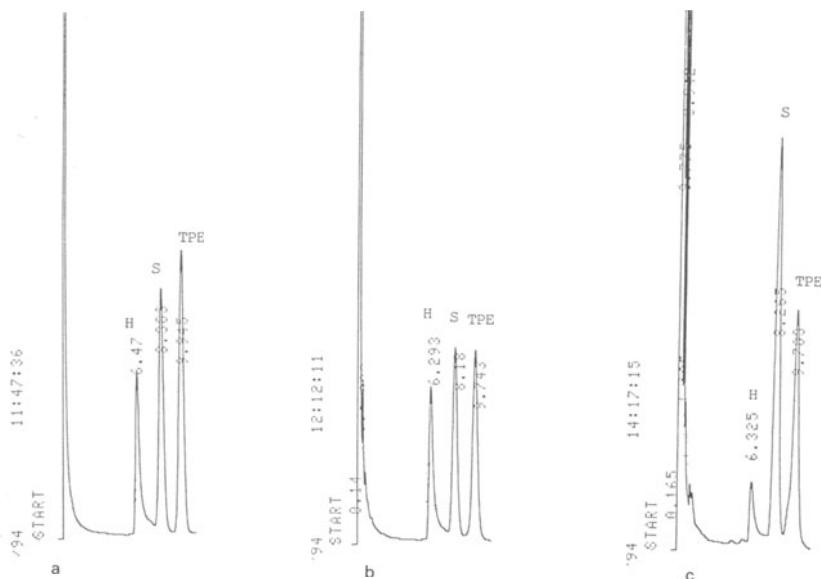


Fig. 4. GLC chromatograms (Luanratana 1995). **a** Reference standards and internal standard. **b** Leaf sample of 4-month-old *Duboisia* hybrid grown in Nakorn Pathom. **c** Leaf sample of 10-month-old *D. myoporoides* grown in Nakorn Rachasima. H Hyoscyamine; S hyoscine; TPE tetraphenylethylene

1.3 In Vitro Culture Studies

Extensive studies have been conducted on various aspects of the in vitro culture of *Duboisia* species, i.e., production of alkaloids (Kitamura 1988), protoplast culture and somatic hybridization (Kitamura 1993), and genetic transformation (Mano 1993), etc. In particular, alkaloid production has been studied by many workers (Endo and Yamada 1984, 1985; Kitamura et al. 1985b, 1988). One of our studies involved promoting the production of hyoscine by using a combined effect of proline and protein synthesis inhibitors. Rooted leaves and regenerated plants were cultivated in revised-tobacco (RT) media containing 10 mg/l naphthalene acetic acid (NAA) and 100 mg/l proline plus one of the protein inhibitors: chloramphenicol, cycloheximide, or puromycin. All cultures showed reduction in growth in comparison with controls. After 30 days the rooted leaves cultivated in the media containing 25 mg/l chloramphenicol gave a threefold yield of hyoscine. Further culture suppressed the production of hyoscine. The regenerated plants cultivated in the media plus 50 mg/l chloramphenicol or 4 mg/l puromycin for 60 days showed a twofold increase in hyoscine production (Luanratana 1991).

2 Micropagation of *Duboisia* spp.

The need for micropagation of *Duboisia* plants is obvious. The seeds are hard and tiny, kidney-shaped, and only 1.2–1.4 mg in weight. Normal seed germination rate is low, 8% being regarded as satisfactory for *Duboisia* species (Kelenyi 1949). This and early loss of seed viability are the two major factors limiting commercial plantation development (Kukreja and Mathur 1984). Experimental germination in Japan gave a yield of only 2% in 50 days. Pretreatment with gibberellic acid at 100 ppm for 48 h reduced the onset of germination to 18 days with a duration of 40 days. The germination rate was increased to 50% (Ikenega and Ohashi 1973). Furthermore, the plants derived from seedlings did not show vigorous growth and half died 1 year after planting (Ikenega et al. 1978). In our studies the seedlings died 6 months after planting in Chantaburi. Experimental cultivation in India grew plants from seeds which took 11–14 weeks before planting in the field was possible (Singh et al. 1985). In addition to the above, other reasons for using tissue culture are to reduce variation among the progeny derived from seedlings, and if somaclonal variation occurs, to select high alkaloid yielding and genetically stable clones. Another advantage is that thousands of plants could be obtained per explant per year (Kukreja et al. 1986).

2.1 *Duboisia myoporoides*

Plant regeneration of *D. myoporoides* R. Br. from callus was first reported by Kitamura et al. (1980). The callus tissues were obtained from stem explants of

a mature tree on MS medium (Murashige and Skoog 1962) supplemented with either of these additions: 0.01 mg/l kinetin and 0.5 mg/l 2,4-D, or 0.01 mg/l kinetin and 10 mg/l NAA. The best shoot bud formation was obtained with MS medium supplemented with 5 mg/l benzyladenine (BA) under a light or dark/light cycle. When the shoots developed five to six leaves, they were then transferred to medium supplemented with 5 mg/l IBA for 2 weeks. After subsequent transfer onto basal medium roots were formed within 2 weeks. The plants were nourished in pots under glasshouse conditions after 1 month in MS media without plant growth substances (Kitamura 1988).

Later, isolation of protoplasts from cell suspension cultures of *D. myoproides* was reported. Callus formation could be induced from protoclony by reduction of osmoticum concentration and nutrient. Shoot differentiation could be obtained on MS medium containing 5 mg/l BAP under light. The shoots were cultured on MS medium containing 5 mg/l IBA for 2 weeks and transferred to basal solid MS medium. After 1–2 months following root formation, the plantlets could be transferred to pots containing sterile sand and peat moss (1:3 v/v) and kept under high humidity for 2 weeks (Kitamura et al. 1989).

Stem node explants could also be used for micropagation. It took 7 weeks to the stage of regenerated plant and another 8 weeks in a glasshouse to harden off (Kukreja and Mathur 1984). Later, direct regeneration of the plants from leaves without the interference of callus formation was suggested as a preferred method for clonal propagation. This would ensure more genetic uniformity and retention of morphogenetic potential than is possible by going through the intervening callus phase. It was noted that a combination of an auxin and a cytokinin was essential to induce shoot bud regeneration and the natural auxin IAA was superior to NAA and IBA. Shoot organogenesis could be obtained directly from the foliar explants cultivated on MS media supplemented with 3 mg/l kinetin and 0.5–1 mg/l IAA. The complete plants were obtained by cultivating the shoots on MS media supplemented with 0.5 mg/l NAA for 7 weeks. The entire process of producing plants up to the glasshouse stage took 4 months. The plants were grown to maturity in the field with a survival rate of 80–90% (Kukreja et al. 1986).

Tissue culture studies in Thailand started from a cutting-derived plantlet, a gift from Dr. W.J. Griffin (University of Queensland, Australia) in 1986 (Fig. 2A,B). Both leaf and stem explants were used for callus induction, the stem explants with buds also gave rise to shoots. Various combinations of auxin and cytokinin were tried for callus initiation; from 180 explants yielded in 22 days, 18 callus lines from leaf explants and 13 callus lines from stem explants (Table 1). The RT medium supplemented with 1 mg/l 2,4-D, 1 mg/l IAA, and 0.6 mg/l BAP was best for callus induction from the explants (Fig. 2C; Luanratana 1988). There was no difference between callus from leaf and stem explants with respect to shoot differentiation (Fig. 2D) and plant regeneration. Moreover, shoot formation could be induced directly from regenerated leaves placed adaxially on RT medium containing 4 mg/l NAA and 4 mg/l BAP. Shoots 1 cm in height were selected and transferred to RT media without any plant growth substances to promote shoot growth. After 4 weeks the shoots

Table 1. Initiation of *D. myoporoïdes* tissue culture on RT medium containing various combinations of plant growth substances. (Adapted from Luanratana et al. 1990)

Explant	Condition	Plant growth substance ^a (mg/l)	Tissue induction	Duration (days)	Callus line
Stem	Light	I 1	Shoot	43	1
	Dark	I 1	Callus	22	1
	Light	N 5	Callus, shoot	22, 28	1
	Light	N 0.05 B 0.1	Shoot	22	1
	Light	N 0.05 B 0.5	Callus, shoot	22	3
	Light	N 4 B 4	Callus, shoot	22	2
	Light	D1 I 1 B 0.6	Callus	22	4
Leaf	Dark	I 3	Root	22	2
	Light	N 5	Callus	22	2
	Light	N 0.5	Callus	22	1
	Light	N 0.01 B 0.5	Callus	22	1
	Light	N 0.05 B 0.5	Callus	22	2
	Dark	N 0.05 B 0.1	Callus	22	1
	Light	D 1 I 1 B 0.6	Callus	22	4
	Light	N 4 B 4	Callus, shoot	22, 43	4
	Dark	N 4 B 4	Root	22	1

^aI, indole acetic acid; N, naphthyl acetic acid; B, 6-benzylaminopurine; D, 2,4-dichlorophenoxyacetic acid.

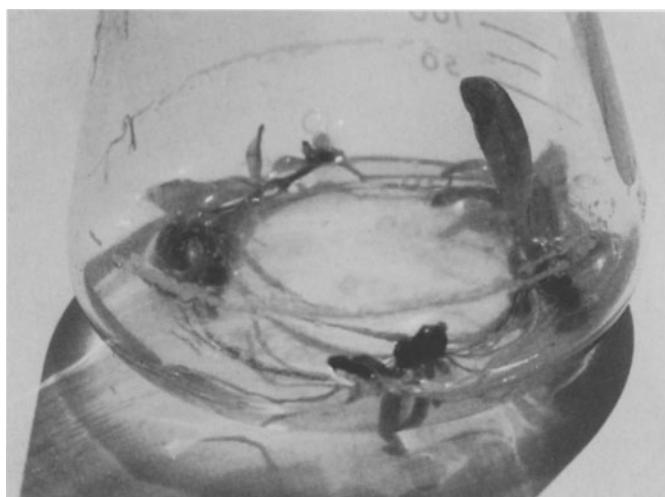


Fig. 5. Root organ culture showing development of green shoots perpendicular to the root pieces

normally developed to 3–4 cm in height and were ready for transfer to the rooting media (Fig. 2E).

Shoot induction was also observed when pieces of root were cultured in a liquid B5 medium for 3 months under light at a shaker speed of 60 rpm (Fig. 5). A green spot developed on the root piece which later gave rise to a normal

Table 2. Percentage of root formation on shoots of *D. myoporoides* (DM) and *Duboisia* hybrid (DH) on MS media containing various concentrations of auxin

Auxin	0.5 ^a	1	2	3	4	5
IBA						
DM ^b	20	15	40	45	65	55
DH ^b	40	40	45	50	55	60
IAA						
DM ^b	40	40	50	55	55	60

^a mg/l.^b Total number of 25 bottles.

shoot at an angle perpendicular to the root. All root pieces gave shoot differentiation, one shoot per root (Luanratana 1990a).

Roots could be induced from differentiated shoots cultured on RT media supplemented with various concentrations of auxins. IBA, IAA, and NAA were normally used at a concentration of 2–3 mg/l; the onset of rooting varied from 1–4 weeks (mode = 2 weeks). It was shown that 10 mg/l NAA could induce root growth from differentiated leaves (Luanratana 1991). Other media could also be used for root promotion, i.e., B5 and MS supplemented with 5 mg/l IBA and 0.5–5 mg/l IBA, respectively. The best medium for root promotion from shoots was MS containing 4 mg/l IBA, which gave 65% rooting within 2 weeks (Luanratana 1994). IAA could also be used at 5 mg/l giving 40% rooting within 2 weeks (Table 2). The advantage of IAA over IBA was the absence of callus. The regenerated shoots could be treated as normal cuttings and propagated by the conventional techniques. A high rooting rate (86%) was obtained, an average from three experiments involving a total of 300 shoots (Luanratana 1990a).

In the transplantation process the regenerated plants were transferred to a pot mixture (50:50 sand:rice ash) and kept in polythene bags under a normal environment or in a growth chamber under light for 2 weeks. The plants were then slowly exposed to normal environment by cutting the bag gradually for another 2 weeks (Fig. 2F). The best temperature range for transplantation is 26–28°C at which almost all of the plants survived; a higher temperature reduces the survival rate (Luanratana 1994).

2.2 *Duboisia leichhardtii*

The callus could be initiated from leaf, stem, and flower buds on B5 media supplemented with 5×10^{-5} M NAA and 5×10^{-5} M BAP. Shoot differentiation could be induced on B5 media containing 10^{-5} M BAP under light for 4 months. The regenerated plants could be obtained when differentiated shoots were transferred to B5 medium containing 10^{-5} M IBA (Endo and Yamada 1984).

2.3 *Duboisia* Hybrid

The micropropagation of a commercial *Duboisia* hybrid has also been successful (Lin and Griffin 1992). A hybrid apparently derived from a male *D. leichhardtii* and a female parent of the Northern *D. myoporoides* variety is cultivated for commercial purposes in Australia. Seeds were better explants than the shoot tips. There was 73% success in the initiation of callus from seed explants in 1 month in comparison to 51% when shoot tips were used. Both auxin and cytokinin were necessary for the growth of callus as had been found earlier with *D. myoporoides* (Kitamura 1988). The callus obtained from seeds also showed consistent, vigorous growth, while callus from shoot tips finally turned brown and exhibited poor growth, but yielded better shoot differentiation. Based on the latter property a general procedure has been recommended for plant regeneration from shoot tip callus, the highest induction frequency being 18% (Lin and Griffin 1992). The entire process of producing plants up to the hardening off stage took 30 weeks from the initiation of callus from explants or 24 weeks from stock callus. The hardening off before planting in the field took 4–8 weeks.

Hybrid studies in Thailand used callus derived from cuttings of a hybrid plant imported from Australia. Callus was induced from several hundreds of leaf and stem explants on the RT media containing 4mg/l NAA and 4mg/l BAP. The RT medium supplemented with 1mg/l 2,4-D, 1mg/l IAA, and 0.6mg/l BAP and 1/2 MS medium containing 10mg/l NAA and 0.1mg/l BAP could also be used for callus induction. There was a high contamination rate which was the result of transportation conditions and time length. However, the callus obtained from imported cuttings showed retardation in growth and always developed a dark brown color after each transfer to a fresh medium (Luanratana 1988; Lin and Griffin 1992). This was due to the fact that tissue cultures of woody plants are often faced with the problem of in vitro production of polyphenol exudates. Various methods were used to overcome this problem (Compton and Preece 1986). In our experiments some green callus was developed on top of the brown callus. This suggested that the basal medium (MS) could have contained too strong a salt concentration. Reduction thereof to one-half, one-quarter, or one-eighth gave good callus; however, upon further subculture at the latter two concentrations the callus growth was reduced, so only 1/2 MS was used. The addition of ascorbic acid or activated charcoal at a concentration of 0.1% effectively eliminated the darkening of callus. Further subculturing in the same medium showed that activated charcoal was better than ascorbic acid because growth was not retarded. Thiamine at a concentration of 0.2% also prevented browning of callus and slightly increased the fresh weight of callus. Amino acids, ornithine, phenylalanine, and proline, at a concentration of 0.1%, increased the growth and weight of callus (Luanratana 1989b). The severity of browning after subculture was reduced over a long period of time. The tissues tended to produce lower concentrations of phenols than newly established cultures. At present, the tissue can be subcultured on MS medium containing a full salt concentration with minimal browning.

Recently, callus has been easily induced from the field-grown plant. The fourth and fifth leaves of a 5-month-old plant were subjected to surface sterilization within a few hours after harvesting. Each leaf was cut into three pieces yielding 102 leaf explants and placed on MS medium containing 1 mg/l NAA and 2 mg/l BAP. Of these explants, 45 started to develop callus or became enlarged and there was no brown coloration.

Some combinations of NAA and BA induced shoot differentiation: 0.01 mg/l NAA and 5 mg/l BAP, 0.1 mg/l NAA and 10 mg/l BAP, and 0.1 mg/l NAA and 15 mg/l BAP. The latter gave the highest percentage (18%) of shoot differentiation (Luanratana 1989b). The use of two cytokinins improved shoot differentiation. Several small shoots could be induced on 1/2 MS containing 2.5 mg/l BAP and 12.5 mg/l 6-dimethylallylaminopurine (2-IP) (22%) (Luanratana 1990b). The media RT or MS, N1B2 and N2B3 could be used to promote shoot growth from shoot buds or callus (90%). Promotion of single shoot growth was carried out on basal MS media supplemented with 0.05 mg/l NAA and 1 mg/l BAP. Differentiated shoots could be induced to form a complete plant by cultivation in MS medium containing 0.5–5 mg/l IBA (40–60%). The latter concentration gave the shortest onset of rooting and most of the shoots developed roots within 2–4 weeks; a few developed roots after 7 days of subculturing. The transplantation process could be carried out in the same way as with *D. myoporoides*. Later experiments revealed an increase in percentage of root formation with increasing time; as high as 68% could be obtained by the end of the sixth week (Table 3).

Genetic engineering is currently playing an important role in plant improvement and in producing novel plants (see Bajaj 1995). The hybrids between *D. hopwoodii* and *Nicotiana tabacum* could be obtained through protoplast fusion (Endo et al. 1988, 1991). Ten subclones of calli derived from a single somatic hybrid cell were cultured for 3 years. Their genetic makeup was expressed by DNA content, chromosome constitution, and peroxidase isozymes. It was found that the mean DNA content reduced with time due to the elimination of tobacco chromosomes. The regenerated shoots also showed different morphology. The relation between nuclear genome composition in shoots of hybrids and alkaloid composition in hybrid plantlets was shown. The line having very weak *Duboisia*-specific bands contained nicotine but neither hyoscyamine nor hyoscine; its morphology also resembled that of *Nicotiana*. The alkaloid metabolism was also studied in the shoot cultures of these hy-

Table 3. Percentage of root forming shoots of *Duboisia* hybrid on MS media containing 5 mg/l IBA with increasing time

Time (weeks)	No. of bottles with roots	Percentage of bottle with roots
2	67 ^a	31.90
6	144 ^a	68.57

^aTotal number of 210 bottles.

brids. In general, the hybrid plants showed activities specific to both *Nicotiana* and *Duboisia* species. Some were able to produce tropane alkaloids after root formation (Endo et al. 1991).

Somatic hybrids between *D. leichhardtii* and *D. myoporoides* were obtained by protoplast fusion. The identification of the somatic hybrids could be accomplished at the callus stage. This was done by analyzing the restriction fragment length polymorphism (RFLP) profiles using non-radioactive rice ribosomal DNA as a probe. With this method *D. leichhardtii* and *D. myoporoides* were easily distinguished by the presence of the bands specific to each species. The hybrids contained both parent bands (Mizukami et al. 1993).

3 Production of Alkaloids in Regenerated Plants

It is generally accepted that the alkaloids in *Duboisia* species are synthesized in the root and transferred to the leaf. Hashimoto et al. (1990) showed that proximity of alkaloid-synthesizing cells to the phloem and the xylem may be important for the transport of tropane alkaloids from the root to the aerial parts of the plant. The production of alkaloids in differentiated tissues and regenerated plants of *D. myoporoides* was studied in comparison with that in seedling and mother plants (Kitamura 1988; Kitamura et al. 1985a,b, 1988). It was concluded that the leaves of the seedlings always contained the main alkaloids throughout the development period but in 1-month-old regenerated plants the roots have higher concentrations.

Our previous studies suggested that the accumulation of hyoscine in the leaves of a hybrid between *D. leichhardtii* and *D. myoporoides* depends on the degree of transpiration in the plants (Luanratana 1982). This resulted in the highest leaf alkaloid content during warmer months and minimal content in winter (Luanratana and Griffin 1980b). This finding confirmed the seasonal variation (Ikenaga et al. 1977b). In contrast, there was no reduction in hyoscine content in glasshouse plants during winter due to controlled temperature (Luanratana and Griffin 1980a). Our studies also showed that there were no significant variations of leaf alkaloids in *D. myoporoides* and *Duboisia* hybrid grown in Thailand under field conditions with a temperature range between 18–39°C throughout the year (Luanratana 1990a). Thus, it would appear that lower temperatures, as experienced in winter in Japan and Australia, are required to reduce the alkaloid accumulation in *Duboisia* leaves.

Transpiration also plays an important part in the accumulation of alkaloids in leaves of the regenerated plants at the early stage of development. In accordance with previous findings (Kitamura 1988; Kitamura et al. 1985a,b), the root of the 1-month-old regenerated plant in a closed system is the organ which accumulates most of the alkaloids. In contrast, under glasshouse conditions, the leaf of 1-month-old plants regenerated from callus accumulated most of the alkaloids, including hyoscine. In an open system (glasshouse conditions) when air movement was present, transpiration was active, there-

fore the alkaloids were translocated from the roots and accumulated in the leaves. In a closed system plants are cultivated under high humidity which inhibits the transpiration process, so that the alkaloids synthesized by the roots cannot move to the leaves. However, over a longer period of time, the humidity in the closed system was reduced, leading to activation of the transpiration process as evidenced by an increase in alkaloid content in the shoot (Trakoonhan et al. 1990).

Differentiated shoots produced neither hyoscine nor hyoscyamine (Griffin 1979; Yamada and Endo 1984), whereas rooted leaves did; the latter have been suggested as another potential source for in vitro production of hyoscine, since they have a higher mass of alkaloid producing tissue compared with 1-month-old regenerated plants (Luanratana 1991).

4 Conclusion

Duboisia, which is a source of tropane alkaloids, can be propagated by tissue culture. The regenerated plants of *D. myoporoides* in our studies showed a higher alkaloid content than the mother plant. The most critical period is the transplantation period, which is usually unreported. Micropagation for commercial purposes would need an almost 100% success rate in the transfer from aseptic conditions to normal environment. This was achieved with *D. myoporoides* at 26–28°C. The best medium for micropagation should be the one which not only gives high rooting rates but also healthy regenerated plants with a normal life span. Our observations over 2 years showed an 88% survival rate of *D. myoporoides*, and 2 out of 30 plants yielded flowers. Another method of obtaining regenerated plants was conventional propagation of the differentiated shoots, which yielded an 86% success rate. This would reduce time and cost of production. However, the growth of these plants under field conditions should be assessed before judging its applicability.

The regenerated plants of *Duboisia* hybrid could be obtained at 68% from differentiated shoots. Transplantation was also critical, but could be done with great care. The plantlets required a longer period than *D. myoporoides* to adjust to normal environment. Growth under field conditions remains to be studied.

5 Protocol

The recommended methods for micropagation are as follows:

1. *D. myoporoides*. The explants could be either leaf or stem; the derived callus could be used for further differentiation. Callus induction could be best obtained in 4 weeks in RT medium containing 1 mg/l 2,4-D, 1 mg/l IAA, and 0.06 mg/l BAP under light. Upon subculturing to RT

medium supplemented with 4 mg/l NAA and 4 mg/l BAP under light, the callus yielded multiple shoots in 4 weeks. Single shoots were selected and subcultured to basal RT medium under light to promote shoot growth for 4 weeks. Shoots 3–4 cm in height could be induced to form complete plants by transferring them to MS medium supplemented with 4 mg/l IBA under light for 4 weeks. Transplantation could be best achieved under high humidity in a growth chamber at 26–28°C or keeping the plantlets in closed polythene bags in the shade for 2 weeks. Adaptation to the normal environment should be done slowly and carefully by making holes in the polythene bags during the second fortnight.

2. *Duboisia* Hybrid. Leaf explants are preferred to stem explants and callus induction should be done promptly after harvesting. Callus induction and multiple shoot development could be induced on MS supplemented with 1 mg/l NAA and 2 mg/l BAP under light for 4 weeks. Single shoot promotion could be done on MS media with 0.5 mg/l NAA and 1 mg/l BAP under light for another 4 weeks. The regenerated plants (68%) could then be obtained on MS media supplemented with 5 mg/l IBA under light for 6 weeks. Transplantation was also carried out using the above method for *D. myoporoides*, but needed more time and greater attention.

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II.2 Microppropagation of *Matricaria chamomilla* L. (Camomile)

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1 Introduction

1.1 Botanical Aspects, Distribution, and Importance

Matricaria chamomilla L. (English name: German camomile) is a biennial herb belonging to the family Asteraceae, which includes many species of medicinal herb, such as pyrethrum (*Chrysanthemum cinerariaefolium* Visiani), yarrow (*Achillea millefolium* L.), wormwood (*Artemisia absinthium* L.), etc. The plant called “Roman camomile” is a different species, *Anthemis nobilis* L. The stems of *Matricaria chamomilla* are branched 30–60 cm in height, with alternating pinnatisect leaves. The flowers bloom from May through October (Fig. 1). Each solitary capitulum, which looks like one flower, comprises aggregated dimorphic flowers, namely, yellow disk florets surrounded by one layer of white ray florets. They are found in Europe, the Middle East, Siberia, and North America, and are also cultivated as agricultural products or ornamental plants in gardens. The dried capitula of this plant have a sweet fragrance and a slightly bitter flavor, and have been popularly used as folk medicine, tea, and a bath additive since olden times. In Europe, especially in Germany, camomile tea is commonly taken as a home cure-all for headaches, stomachaches, colds, etc. Much research on its pharmacological compounds has found that the secondary metabolites, such as sesquiterpenoids, coumarin, and flavonoids are synthesized (Isaac 1974; Greger 1977; Verzar-Petri et al. 1979).

1.2 Conventional Propagation and In Vitro Studies

Usually camomile is easily propagated from seeds. This plant is characteristically quite strong and it branches very well. Many achenes are set in each capitulum by open pollination, and it germinates vigorously, growing even from seeds falling spontaneously on the ground.

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Fig. 1. *Matricaria chamomilla* L. (Photograph kindly provided by Dr. S. Izumi)

Since propagation from seeds is relatively easy, applications of the tissue-culture techniques to *M. chamomilla* are mainly focused on particular purposes, such as the efficient production of useful secondary metabolites, clonal propagation of superior individuals in uniform genotypes, mutation breeding, and materials for cryopreservation, etc. Several papers have reported on the production of useful secondary metabolites (Reichling and Becker 1976; Szoke et al. 1977a,b, 1978; Bisson et al. 1983; Reichling et al. 1984; Reichling and Beiderbeck 1991; Hirata et al. 1993), clonal mass propagation (Takano et al. 1991), and cryopreservation (Dietrich et al. 1990; Cellarova et al. 1992). The cultured tissues or cells used in these studies were calli, suspended cells and masses of shoot primordia, and whole plants were also used as controls. Among these tissues, a mass of shoot primordia is considered to be one of the most appropriate for the purpose of vegetative propagation of plants. The advantages of the shoot primordia include high growth rate, high regenerative potential and genetic stability (Tanaka and Ikeda 1983).

2 Microppropagation

2.1 Methodology

2.1.1 Induction of Shoot Primordia

1. *Culture Medium.* Prepare an appropriate amount of an MS liquid medium (Murashige and Skoog 1962), supplemented with 2 mg/l 6-benzylaminopurine (BAP) and 3% (w/v) sucrose. Adjust the pH to 5.7–5.8. Pour approximately 20 ml of the medium into each of the test tubes, which are 30 mm in diameter and 200 mm in length. Cover the mouth of the test tubes with double-layered

sheets of aluminum foil for closure. Sterilize them in an autoclave at 121 °C for 15 min.

2. *Inoculation of Shoot Tips.* It is desirable that the material plants are healthy and show vital growth. Upper parts of the shoots, approximately 1 cm long containing growth points, are cut from the plants. For surface sterilization, the explants are first immersed in 0.1% (w/v) benzalkonium chloride for 5 min, then immersed in 1–2% (w/v) sodium hypochlorite for 5 min (bleach or a disinfectant solution for household use can be used; these usually contain 6–10% active constituents). The samples are then rinsed twice with sterilized water. The shoot tips, having a meristematic dome and two-leaf primordia, are aseptically excised from the explants with tweezers and a scalpel under a stereoscopic microscope. They are then inoculated into the liquid medium in the test tube.

3. *Culture Conditions.* The shoot tips are incubated in a gyratory drum vertically rotating at 2 rpm, under a continuous illumination of 9000 lx at 22 °C (Fig. 2). This physical condition for culture is an important point for the induction of masses of shoot primordia.

2.1.2 Subculture of Masses of Shoot Primordia and Regeneration

1. *Subculture.* A mass of shoot primordia forms from a shoot tip 1–2 months after inoculation. As it propagates, it spontaneously separates into several masses. These are subcultured in a fresh medium every 2 weeks.
2. *Regeneration Medium.* A one-fourth strength liquid MS basal medium



Fig. 2. Gyratory drum for the culture of shoot primordia

supplemented with 1% (w/v) sucrose is prepared. Adjust the pH to 5.7–5.8. With regard to supporting material, either of the following two methods are worthy of consideration. However, the second method, by which better regeneration results were obtained, should be preferred.

- a) Agar. Agar 0.9% (w/v) is added to the liquid medium described above, and the medium is heated in a boiling water bath until the agar dissolves completely. An appropriate amount of the agar medium is then poured into the culture pots, and autoclaved.
 - b) Ceramic wool mats (2.5 cm thick, Shin-Nihon Kagaku Co., Japan) or the equivalent. The ceramic wool is cut into blocks 5 × 2.5 × 2.5 cm, and two blocks are placed in the culture pot so that the ceramic fibers run vertically. Liquid medium is poured into the pot to half the height of the ceramic wool, and autoclaved.
3. Cut the mass of shoot primordia into small pieces (1–2 mm in diameter) with a pair of tweezers and place them onto the medium.
 4. Incubate statically under 3000–5000 lx illumination for 16 h/day, at 22 °C. In 6 weeks, each shoot primordium will have grown into several small plantlets ready for transfer to soil.

2.1.3 Acclimatization and Transfer to Soil

1. Take out the well-grown and rooted plantlets, together with the ceramic wool. Thoroughly rinse the liquid medium from the ceramic wool mat with running tap water.
2. Transplant the plantlets to clean, heat-sterilized soil (e.g., Vermiculite: Perlite = 1:1), together with the ceramic wool.
3. Acclimatize the regenerated plants by carefully maintaining a high humidity environment for 1 week in the shade, and then by gradually reducing the humidity and increasing exposure to light. After sufficient acclimatization, the plantlets can be grown in the same conditions as in vivo plants.

2.2 General Characteristics of Shoot Primordia

The tissue-cultured masses of shoot primordia were found during an experiment for the development of a mericlon system in an annual Asteraceae, *Haplopappus gracilis* (Tanaka and Ikeda 1983). Following this, the cultured tissues with similar characteristics were successfully induced in other plant species. The common morphologies and regeneration among these shoot primordia, which are different from calli or multiple shoots, are:

1. The smallest unit of the tissue is a nodular dome with a green, pale green, or pale yellowish-green color, which can be identified as one shoot primordium. A mass of shoot primordia, containing many shoot-primordial domes is induced from a shoot tip. Each dome produces new daughter domes when it grows to a certain size. The shoot primordia propagate by this

growing cycle, without further differentiation. Approximately four domes are newly produced per shoot primordium in a week, which leads to vigorous propagation of the masses.

2. They have a high regeneration capacity. One shoot primordium regenerates into multiple shoots, involving the production of new shoot primordium domes. Then they form adventitious roots and grow into whole plants.

2.3 Medium Composition Appropriate for the Induction of Shoot Primordia

The conditions of a medium that is appropriate for the induction of shoot primordia were examined by an evaluation of ten hormonal combinations: shoot tips were inoculated in the MS basal media supplemented with hormone combinations of NAA (0, 0.02, 0.2, 2, and 4 mg/l) and BAP (0.2 and 2 mg/l). Table 1 shows the results of a morphological observation of the tissue induced under each condition 3–4 weeks after inoculation. Depending on the hormonal condition, different types of tissues were observed, such as a callus, a mass of shoot primordia, and multiple buds. There was a clear correspondence between the hormone concentration and the degree of differentiation of the induced tissue, by the interaction of the two growth regulators. The tissues selected as shoot primordia were induced in the medium with 2 mg/l BAP (Fig. 3A,B). They were masses 1–2 cm in diameter, with many green domes. The propagating style of the camomile shoot primordia was similar to that of *Haplopappus*: a dome developed to a certain degree produced new domes on its surface, and these propagated by this continuous cycle of new dome production. Histological observation revealed that the shoot primordium domes

Table 1. Morphology of the tissues induced from shoot tips of *M. chamomilla* in MS media modified with a combination of NAA and BAP. (Takano et al. 1991)

NAA (mg/l)	BAP (mg/l)			
		0.2	2	
0	S + C ^a	1/4 ^b	S + SP	1/4
	C	1/4		
	D	2/4	D	3/4
0.02	C	1/4	S + C	2/4
	D	3/4	D	2/4
0.2	S + C	1/4	SP + C	1/4
	C	1/4	C	1/4
	D	2/4	D	2/4
2	C	1/4		
	D	3/4	D	4/4

^a S, shoots; C, calli; SP, shoot primordia; D, dead.

^b Number of tissues induced/number of shoot tips inoculated.

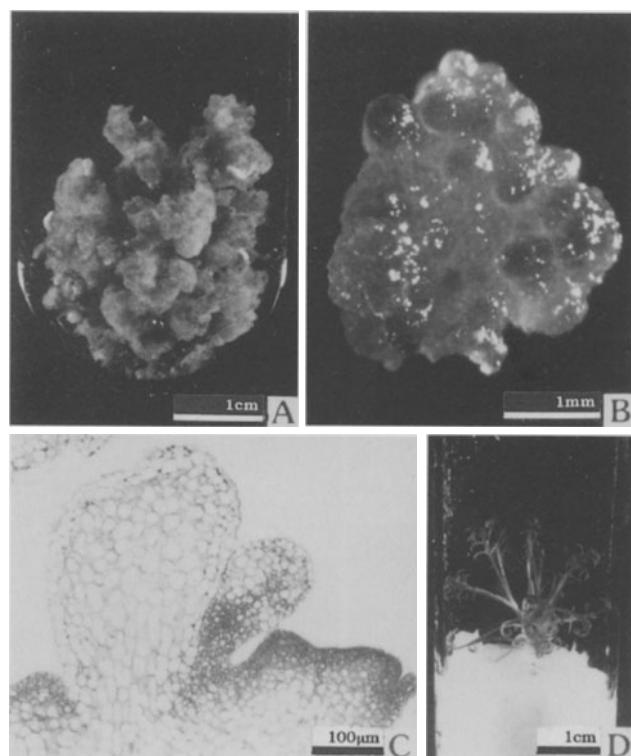


Fig. 3. Shoot primordia and the regenerated plantlet of *Matricaria chamomilla*. **A, B** Shoot primordia; **C** cross section of the shoot primordia; **D** a regenerated plantlet from shoot primordia. (Reproduced from Takano et al. 1991)

which organized the masses were mainly classified into two developmental stages (Fig. 3C): (1) the primary shoot primordium referred to relatively small domes, which consisted of small plasmatic cells; (2) the secondary shoot primordium referred to the larger domes, which had one epidermal layer and larger vacuolated cells inside. This constitution of domes in a mass was also similar to that in *Haplopappus* shoot primordia. The growth of the camomile shoot primordia was at such a rate that approximately 100 mg of fresh shoot primordia was propagated into 3 g in 2 weeks, after which propagation stopped and entered a steady period. Subculturing at more than 2-week intervals greatly prevented the shoot primordia from maintaining good conditions and make them turn brown in color. Thus subculturing every 2 weeks was appropriate for maintenance of the camomile shoot primordia.

2.4 Regeneration of Plantlets from Shoot Primordia

The camomile shoot primordia could be easily regenerated into plantlets on a conventional agar-solidified medium, as was performed on the shoot primor-

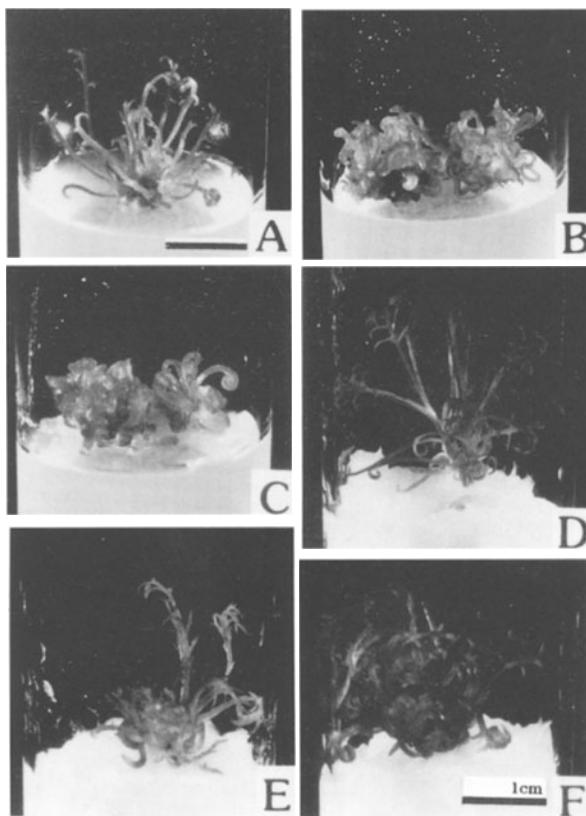


Fig. 4. Plantlets regenerated from shoot primordia of *Matricaria chamomilla*. **A-C** Solidified by agar; **D-F** supported by ceramic wool. Media contain quarter strength MS, 1% sucrose, and 0, 0.02, and 0.2 mg/l NAA in **A** and **D**, **B** and **E**, and **C** and **F**, respectively.

dia of other plant species. As to the needs of mass production of the regenerated plants, we further examined the conditions for obtaining regenerants relative to the following factors: (1) Concentration of sucrose and growth regulators: 18 conditions of an MS agar medium with various combinations of sucrose (1, 2, and 3%), NAA (0, 0.02, and 0.2 mg/l), and BAP (0, 0.02 mg/l) were evaluated. The best results were obtained when 1% sucrose was supplemented and without any growth regulator (data not shown). (2) Concentration of the basic mineral nutrition and the supporting material besides the hormonal conditions: the various strengths of an MS basal medium, agar and ceramic wool as a supporting material, and the hormonal conditions were examined in combinations (Fig. 4, Table 2). The conditions greatly affected the rate of root formation, while no remarkable difference was observed in shoot formation ability. The entire evaluation of these results and the quality of the regenerated plants obtained (e.g., sturdiness, rapid growth, etc.) indicated that the best combination for obtaining regenerated plants was a quarter

Table 2. Effects of the supporting materials, phytohormone and the strength of MS basal media for the regeneration from shoot primordia of *M. chamomilla* after a 12-week culture. (Takano et al. 1991)

Base layer	BAP (mg/l)	NAA (mg/l)	Basic media				\pm^b
			1/4 MS	1/2 MS	MS		
CW	0	0	4.0 ^a	+++ ^b	9.5 ^a	++ ^b	8.0 ^a
		0.02	3.0	+++	6.0	++	8.0
		0.20	3.0	+++	5.0	+++	14.0
	0.02	0	8.5	+++	10.0	+++	9.5
		0.02	4.0	+++	9.5	++	7.5
		0.20	2.5	+++	2.5	+++	5.5
Agar	0	0	7.5	+++	8.0	\pm	10.0
		0.02	8.5	+++	9.0	\pm	8.5
		0.20	2.0	++	5.0	—	3.0
	0.02	0	11.0	+	8.5	++	9.5
		0.02	8.5	+	4.0	\pm	6.5
		0.20	0.0	—	3.5	\pm	6.0

CW: ceramic wool

^aMean number of shoots regenerated from one 1.5-mm piece of shoot primordia mass.^bRoot formation of the regenerated shoots: +++ all; ++ most; + half; \pm a few; — none of the shoots regenerated roots.

strength of the basal medium, hormone-free and ceramic-wool supporting. Added to the efficiency for regeneration itself, using ceramic wool as the supporting material had great advantages for acclimatization of the regenerated plants. The liquid medium could be thoroughly rinsed from the regenerants using running tap water and they were transplanted to the pots, together with the ceramic wool mats. The advantage of this method was to avoid physical damage to the roots, infection with fungi and bacteria, in addition to saving both time and labor. Although the cost may be to some degree higher than that of agar, the regeneration system using ceramic wool will contribute to the automated mass production of young plants in the future. Thus, ceramic wool and rock wool should be further studied as a supporting material for the regeneration of cultured tissues.

2.5 Chromosomal Stability of Shoot Primordia and Regenerants

Camomile has a chromosome number of $2n=18$, which is a usual number among Asteraceae plants. We examined the chromosomal stability of the tissue-cultured shoot primordia after subculture for 4 months and the regenerated plants (Table 3). The shoot primordia and the root tips of the regenerants showed $2n=18$ at frequencies of 99 and 100%, respectively. Although time-lapse observations of the metaphase cells were not performed, the shoot primordia could be expected to maintain a chromosomal stability for at least

Table 3. Chromosome counts in the parental plant, shoot primordia, and regenerated plants in *M. chamomilla*. (Takano et al. 1991)

Cell source	Total cells observed	Number of cells and (%)	
		2n=18 (2x)	2n=36 (4x)
Root tips of parental plant	100	100 (100)	0 (0)
Shoot primordia	156	155 (99.4)	1 (0.6)
Root tips of regenerated plants	100	100 (100)	0 (0)

Table 4. Lipid components in the oil bodies of *M. chamomilla*. (Hirata et al. 1993)

Compound ^a	Content/wt %	
	Shoot primordia	Seeds
TG	78.7	75.7
1,3-DG	2.8	11.9
1,2-DG	5.1	0.9
2-MG	1.2	1.3
1-MG	3.3	1.1
FA	8.2	8.5
PL	0.7	0.6

^aTG,DG,MG,FA, and PL denote triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acid, and phospholipids, respectively.

a few years through the subculture, as was found in the case of the *Haplopappus* and *Crepis* shoot primordia (Tanaka et al. 1988).

2.6 Formation of Oil Bodies in Shoot Primordia

Camomile plants are known to store pharmacological secondary metabolites, such as terpenoids, in their oil bodies. There may be prospects of producing useful secondary metabolites efficiently by the tissue culture system of the shoot primordia. Hirata et al. (1993) examined the distribution of the oil bodies within the shoot primordium tissue and the lipid components of the oil bodies. By and large in the shoot primordia, the size of the oil bodies ranged from 0.3 to 1.5 mm. Smaller oil bodies (<0.3 mm) were distributed exclusively in the outer layer, while the middle-sized ones (0.5 to 0.8 mm) were mainly concentrated in the inner vascular-like meristematic regions and their surrounding areas. Table 4 shows the comparative results of the lipid components in the oil bodies between the shoot primordia and the seeds. Similar components were found in both the shoot primordia and the seeds, except that the contents of the diacylglycerol were on the whole different.

3 Summary

Mass propagation of *M. chamomilla* L. was achieved by a shoot tip culture. The appropriate conditions of a medium for the induction of shoot primordia were examined by an evaluation of ten hormonal combinations. The camomile shoot primordia showed rapid growth without any further differentiation, keeping a high level of chromosomal stability and regeneration capacity. They contained oil bodies with similar lipid components to those in seeds.

4 Protocol

1. Explant: Shoot tips, each having a meristematic dome with the two youngest leaf primordia, from healthy and vigorously growing plants.
2. Medium: MS liquid medium supplemented with 2mg/l BAP and 3% (w/v) sucrose.
3. Culture conditions: A gyratory drum vertically rotating at 2rpm, continuous illumination of 9000lx, 22°C. Subculture every 2 weeks.
4. Regeneration into plantlets: a one-fourth strength of the liquid MS supplemented with 1% (w/v) sucrose, absorbed with ceramic wool mats. 3000–5000-lx illumination for 16h/day, 22°C.

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II.3 Micropagation of *Sideritis* Species

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1 Introduction

The genus *Sideritis* (family Labiate), with more than 130 species, is composed of annual or perennial herbs and small shrubs, widely distributed in the Mediterranean-Macaronesian area. They are usually calcicolous and heliophilous plants forming part of degraded shrublands in dry and semiarid regions. Because hybridization is a very common phenomenon, even between individuals of different sections, the taxonomy of the genus *Sideritis* is somewhat complex (Heywood 1972; Peris et al. 1990).

Sideritis species are currently used in Spanish folk medicine as antibacterial, antiinflammatory, and antirheumatic drugs (Font-Quer 1978; Barberán et al. 1987). Over the last few years, the phytochemistry and pharmacology of several *Sideritis* species have been extensively investigated. The major secondary products of medicinal interest are essential oils, diterpenes, and flavonoids.

Essential oils of *Sideritis* are stored in glandular hairs, their amount and quality vary according to the species, analyzed organs or tissues, and place and season of collection (Villar et al. 1984a,b, 1985a; Mateo et al. 1983, 1984, 1988). The chief constituents of these oils are monoterpenes (α -pinene, β -pinene, Δ^3 -carene, p-cymene, limonene, and fenchone) and sesquiterpenes (δ -cadinene, α -copaene, β -caryophyllene, and α -curcumene). So far, the major pharmacological interest of *Sideritis* oils is their bactericidal and fungicidal properties (Villar et al. 1985b, 1986).

Sideritis diterpenes, particularly 1-acetyljativatriol, also show a potent antimicrobial activity (Diaz et al. 1988). These compounds seem to be also related to the antiinflammatory properties of *Sideritis*. Thus, the diterpene Borjatriol, isolated from *S. mugronensis* Borja (Rodriguez and Valverde 1973), inhibits carrageenan edema (Alcaraz and Villar 1987) and exerts therapeutic effects on adjuvant arthritis, a model related to the human rheumatoid disease (Villar et al. 1983).

The pharmacological use of *Sideritis* flavonoids has been extensively studied in recent years, with research focusing particularly on their antiinflammatory, antibacterial, and antispasmodic activities (Alcaraz and Hoult 1985; Villar et al. 1985c; Barberán 1986; Barberán et al. 1987; Tomás-Barberán

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and Gil 1990). In addition, a potential use of some *Sideritis* flavonoids in preventing diabetic cataract had also been reported (Tomás-Barberán et al. 1986). Finally, as stated by Tomás-Barberán and Gil (1990), *Sideritis* species can be an important source of cosmetically interesting flavonoids widely used in the treatment of age-induced hyperpigmentation of skin spots.

Most of the species of *Sideritis* are still found growing only in the wild. For this reason, conventional vegetative or sexual practices for large-scale propagation of these plants have not been developed yet. Consequently, methods are not readily available to improve *Sideritis* by conventional breeding techniques. In this context, tissue culture technologies can be of immense help in overcoming some of these limitations by providing methods for the conservation, large-scale multiplication and improvement of these important medicinal plants. The impact of biotechnology in medicinal plants has been recently reviewed and discussed (Bajaj et al. 1988; Constabel 1990; Segura and Pérez-Bermúdez 1992). Our work on *Sideritis angustifolia* Lag. is discussed in this chapter.

2 Micropagation

2.1 Summary of In Vitro Studies

Most of the in vitro studies are related to callus induction and growth, isolation, and culture of callus-derived cells, and plant regeneration from seedling explants, callus, or single cells (Sánchez-Gras and Segura 1987, 1988a, 1989). Mesophyll protoplasts of *S. angustifolia* have been recently isolated and cultured (Carvalho et al. 1993), but plant regeneration has not yet been obtained. Furthermore, Sanchez-Gras and Segura (1988b) and Sánchez-Gras et al. (1990) investigated the effects of the polyamine spermidine on in vitro morphogenesis from primary explants and single cells of *S. angustifolia*. In addition, Garcia-Granados et al. (1994) have described a method for micropropagation of *S. foetens* through tissue culture. These authors also evaluated the diterpene content in the regenerated plants.

2.2 Establishment of Tissue Culture

Most of the material used in tissue culture of *S. angustifolia* came from seedlings obtained by germinating seeds under sterile conditions. Seeds were surface sterilized with 2% chloramine-T and 0.01% Tween 20 for 30 min, rinsed with sterile distilled water, and germinated in Petri dishes on solid medium (0.8% agar in distilled water with 28 μ M GA₃) at 26 \pm 2°C in darkness (Sánchez-Gras and Segura 1987). In some experiments, in vitro regenerated plants were also employed as a source of explants (Sánchez-Gras and Segura

1989). Most of the explants under study, including single cells, were cultured on liquid or agar-solidified MS medium (Murashige and Skoog 1962). For protoplast culture, a modified MS medium with double concentration of Ca^{2+} and the rest of the mineral constituents at half strength was used (Carvalho et al. 1993). The carbon source employed was sucrose.

2.3 Callus Culture

Callus cultures of *S. angustifolia* can be easily obtained from hypocotyl, cotyledon, and root explants cultured on solidified MS medium supplemented with different concentrations of auxins (NAA, IAA, or 2,4-D) and cytokinins (BA or Kin) used either singly or in combinations, but these explants did not respond to MS alone (Sánchez-Gras and Segura 1987). Cell proliferation first became visible at the cut ends within a week of initial culture. Calli were friable and bright green in color when they were induced in media containing IAA or NAA, while those obtained in presence of 2,4-D had a mucous consistency and were yellowish-white in color. Treatments with the highest cytokinin concentrations tended to form compact calli. In hypocotyls and roots, IAA and NAA were more effective than 2,4-D, while for cotyledons 2,4-D was the most active auxin. In relation to cytokinins, BA was always more active than Kin in promoting callus formation, irrespective of the explant source. The efficiency of the auxins on callus formation was generally increased by adding cytokinin to the media.

Experiments conducted using leaf explants, isolated from shoots regenerated from hypocotyl cultures (see Sect. 2.4), revealed that callus induction and growth also required the presence of growth regulators (Sánchez-Gras and Segura 1988). Nevertheless, leaf age and light conditions during culture had a marked effect on the morphogenic capacity of these explants. Thus, with the same growth regulator treatment, young explants showed more callus formation than old explants (Fig. 1). In both cases, preincubation in darkness promoted cell proliferation.

2.4 Shoot Regeneration from Segments of Hypocotyl, Cotyledon, and Root

Factors promoting caulogenesis from hypocotyl, cotyledon, and root explants of *S. angustifolia* were reported by Sánchez-Gras and Segura (1987). The induction of adventitious buds and shoots necessarily required the presence of both auxins and cytokinins and was preceded by an initial callus phase. The time for appearance of buds was about 15–20 days and the intensity of the caulogenic response depended on the type of explant and on the particular growth regulator used. Hypocotyls exhibited the best capability for shoot-bud regeneration (Fig. 2), while roots showed only a minimal response when they were cultured on media supplemented with $0.54 \mu\text{M}$ NAA and 4.44 or $8.88 \mu\text{M}$ BA (Tables 1, 2). In relation to hormonal combinations, balanced or low

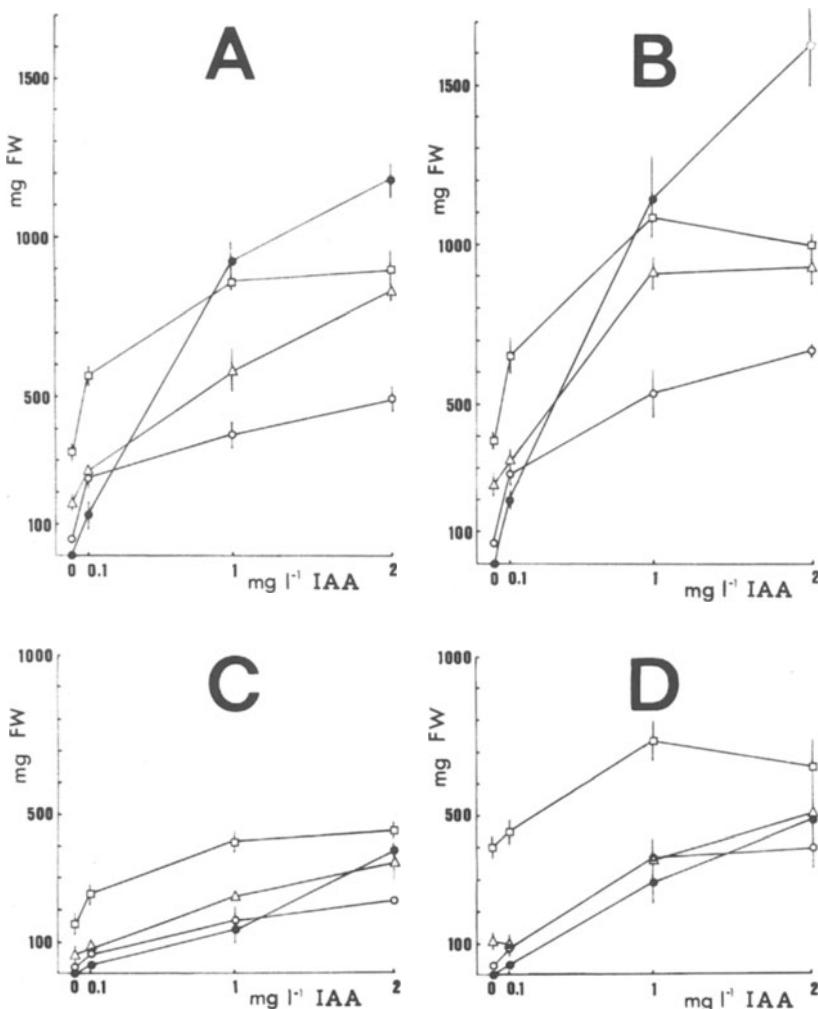


Fig. 1. Effect of different IAA/BA ratios, illumination conditions, and explant age on callus growth (fresh weight, mg/explant) in leaf explants derived from plants regenerated from hypocotyl cultures. Data were obtained after 8 weeks of culture and means \pm SE are from two separated experiments. **A** Darkness preincubation, old leaves; **B** preincubation in the dark, young leaves; **C** photoperiod, old leaves; **D** photoperiod, young leaves. (●) 0 mg l^{-1} BA; (○) 0.01 mg l^{-1} BA; (\triangle) 0.1 mg l^{-1} BA; (\square) 2 mg l^{-1} BA

auxin-cytokinin ratios provided the best results; among these combinations, the most effective were those containing BA and NAA (Table 1). In contrast, all media supplemented with 2,4-D and Kin did not promote shoot-bud regeneration.

An investigation to study the effects of explant age and illumination conditions on caulogenesis from cultured leaf explants was undertaken

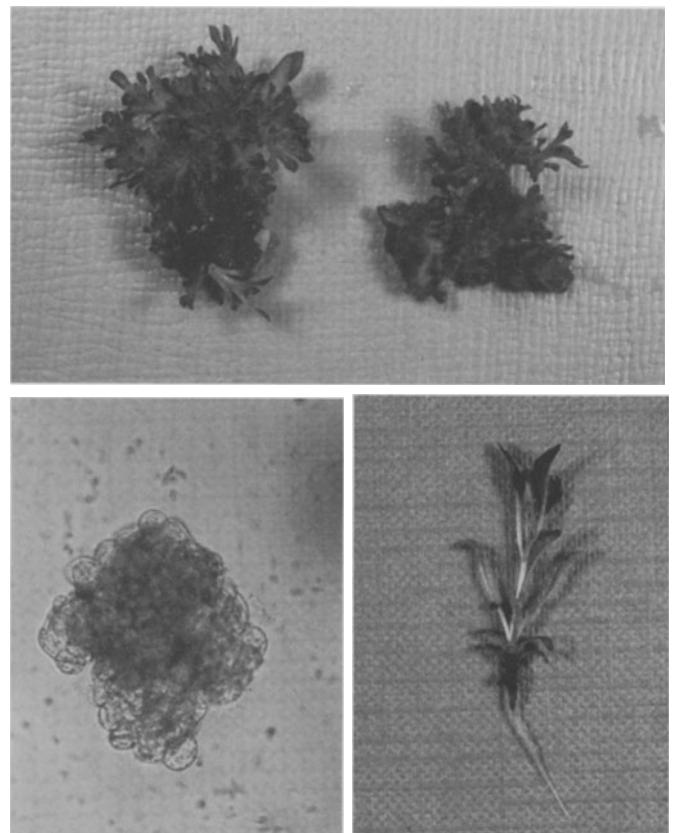


Fig. 2. Shoot-bud development on caulogenic callus derived from hypocotyl explant of *S. angustifolia*

Fig. 3. Microcallus from single cell of *S. angustifolia* after 15 days of culture on MS medium containing $0.5\mu\text{M}$ NAA, $8.8\mu\text{M}$ BA, and 100mg/l CH

Fig. 4. Plantlet obtained by rooting a regenerated shoot of *S. angustifolia*

(Sánchez-Gras and Segura 1989). Three-month-old plants regenerated from hypocotyls cultured in the presence of $5.4\mu\text{M}$ NAA and $4.4\mu\text{M}$ BA were used as a source of explants for the experiment. Explants were excised from the second (old explants) and the fifth (young explants) pair of leaves and cultured on solid medium supplemented with IAA and/or BA. Of the two leaves from each pair, one was cultured in light (16-h photoperiod of 20W m^{-2} from Gro-lux fluorescent tubes) and the other one in the dark. Dark-grown explants were transferred to light after 4 weeks of culture and final data recorded after 8 weeks.

Leaf explants from *S. angustifolia* have the morphogenic potential to regenerate shoots in vitro. Nevertheless, for maximum efficiency of shoot

Table 1. Effect of different NAA/cytokinin ratios on caulogenesis in hypocotyl and cotyledon explants of *S. angustifolia*. Data were obtained after 4 weeks of culture. Means \pm SE are from two separate experiments. (Sánchez-Gras and Segura 1987)

Growth regulators (μ M)			Hypocotyl		Cotyledon	
NAA	Cytokinin		Explants with buds (%)	No. shoot-buds per explant	Explants with buds (%)	No. shoot-buds per explant
0.05	BA	0.04	0	0	0	0
0.05		0.44	17	0.70 \pm 0.20	0	0
0.05		4.44	27	1.30 \pm 0.30	21	1.70 \pm 0.50
0.05		8.88	17	0.40 \pm 0.30	4	0.10 \pm 0.10
0.54		0.04	7	0.10 \pm 0.10	0	0
0.54		0.44	58	1.90 \pm 0.60	0	0
0.54		4.44	37	2.50 \pm 0.20	25	0.20 \pm 0.50
0.54		8.88	17	0.60 \pm 0.10	9	0.10 \pm 0.10
5.40		0.04	14	0.30 \pm 0.20	0	0
5.40		0.44	17	0.90 \pm 0.30	13	0.30 \pm 0.20
5.40		4.44	65	4.30 \pm 1.10	25	0.90 \pm 0.20
5.40		8.88	53	2.90 \pm 0.50	17	0.60 \pm 0.20
10.80		0.04	0	0	0	0
10.80		0.44	0	0	0	0
10.80		4.44	3	0.04 \pm 0.08	0	0
10.80		8.88	17	1.00 \pm 0.30	0	0
0.05	Kin	0.04	0	0	0	0
0.05		0.46	0	0	0	0
0.05		4.65	7	0.70 \pm 1.00	0	0
0.05		9.30	3	0.30 \pm 0.70	0	0
0.54		0.04	0	0	0	0
0.54		0.46	0	0	0	0
0.54		4.65	10	0.20 \pm 0.20	3	0.04 \pm 0.08
0.54		9.30	17	1.10 \pm 0.10	0	0
5.40		0.04	0	0	0	0
5.40		0.46	0	0	0	0
5.40		4.65	7	0.20 \pm 0.20	0	0
5.40		9.30	27	1.60 \pm 0.40	0	0
10.80		0.04	0	0	0	0
10.80		0.46	0	0	0	0
10.80		4.65	0	0	0	0
10.80		9.30	23	1.00 \pm 0.20	0	0

multiplication it became critical to control BA concentration, explant age, and light conditions during culture. Thus, adventitious shoot regeneration was confined to media containing 8.8 μ M BA in combination with IAA (0.5, 5.7, or 11.4 μ M). Young explants were more responsive than old explants and the best results were obtained when the former were precultured in darkness. In addition, old explants failed to regenerate buds when cultured under photoperiod conditions for 8 weeks.

Table 2. Effect of different IAA/cytokinin ratios on caulogenesis in hypocotyl and cotyledon explants of *S. angustifolia*. Data were obtained after 4 weeks of culture. Means \pm SE are from two separate experiments. (Sánchez-Gras and Segura 1987)

Growth regulators (μ M)		Hypocotyl		Cotyledon	
IAA	Cytokinin	Explants with buds (%)	No. shoot-buds per explant	Explants with buds (%)	No. shoot-buds per explant
0.05	BA 0.04	0	0	0	0
0.05	0.44	0	0	0	0
0.05	4.44	0	0	0	0
0.05	8.88	7	0.20 \pm 0.20	0	0
0.57	0.04	20	0.70 \pm 0.30	0	0
0.57	0.44	17	0.80 \pm 0.30	0	0
0.57	4.44	33	2.80 \pm 0.70	3	0.10 \pm 0.30
0.57	8.88	33	1.90 \pm 0.50	3	0.20 \pm 0.40
5.70	0.04	10	0.10 \pm 0.10	0	0
5.70	0.44	13	0.70 \pm 0.70	0	0
5.70	4.44	21	1.40 \pm 0.40	3	0.30 \pm 0.60
5.70	8.88	23	1.40 \pm 0.40	7	0.10 \pm 0.10
11.40	0.04	0	0	0	0
11.40	0.44	7	0.30 \pm 0.30	3	0.10 \pm 0.20
11.40	4.44	11	0.50 \pm 0.40	7	0.50 \pm 1.00
11.40	8.88	27	1.60 \pm 0.30	30	1.70 \pm 0.70
0.05	Kin	0.04	0	0	0
0.05		0.46	0	0	0
0.05		4.65	0	0	0
0.05		9.30	0	0	0
0.57		0.04	0	0	0
0.57		0.46	0	0	0
0.57		4.65	0	0	0
0.57		9.30	3	0.60 \pm 1.20	0
5.70		0.04	0	0	0
5.70		0.46	0	0	0
5.70		4.65	43	2.40 \pm 1.00	0
5.70		9.30	23	2.40 \pm 0.50	0
11.40		0.04	0	0	0
11.40		0.46	0	0	0
11.40		4.65	29	1.80 \pm 0.30	9
11.40		9.30	55	3.10 \pm 1.40	9

Tissue cultures carried out with *S. foetens* (Garcia-Granados et al. 1994), were initiated from nodal sections with two axillary buds. These explants were excised from 5-week-old seedlings obtained from seeds germinated under sterile conditions. The best shoot proliferation rate was achieved when explants were cultured in solidified MS medium supplemented with 0.22 μ M BA. In this study, rooting of regenerated shoots was done *in vivo*.

2.5 Isolation and Culture of Cells and Protoplasts

Single cell cultures were established from callus of *S. angustifolia* (Sánchez-Gras and Segura 1988a). Isolated cells from hypocotyl-derived calli, induced in the presence of $0.5\text{ }\mu\text{M}$ NAA and $8.8\text{ }\mu\text{M}$ BA, were selected to study the effect of different growth regulators on cell proliferation. Cells were cultured at 10^4 cells/ml on solidified MS medium with or without NAA (0.5, 5.4, and $10.8\text{ }\mu\text{M}$) and/or BA (0.4, 4.4, and $8.8\text{ }\mu\text{M}$). In all cases, the medium was also supplemented with 100 mg/l CH. The first division occasionally began within 4–5 days of culture, and cell colonies and microcalli (Fig. 3) were observed after 10–15 days. The higher number of visible calli was obtained in those media including $0.5\text{ }\mu\text{M}$ NAA and 4.4 or $8.8\text{ }\mu\text{M}$ BA (15.4 and 4.2 calli/cm²). Nevertheless, adventitious bud differentiation was only observed on calli grown in the presence of $0.5\text{ }\mu\text{M}$ NAA and $8.8\text{ }\mu\text{M}$ BA.

In order to determine culture conditions that provide a high production of shoots, another experiment was conducted using cell-derived calli grown on medium with $0.5\text{ }\mu\text{M}$ NAA, $8.8\text{ }\mu\text{M}$ BA, and 100 mg/l CH. These calli were transferred to the same medium without CH and maintained for 30 days under a 16-h photoperiod. Subsequently, calli were subcultured to various regeneration media. Different treatments were used depending on the state of differentiation: callus with buds, medium without growth regulators; undifferentiated callus, medium with or without BA alone or in combination with auxins (IAA or NAA). After 15 days of culture, calli showing shoot-buds were transferred to MS medium without growth regulators. This procedure was repeated every 15 days until a 60-day culture period was completed. In each subculture, developed shoots were excised and transferred to medium without growth regulators.

After 30 days of subculture in medium with BA and NAA, 45% of the cell-derived calli showed shoot-bud regeneration. Once the developed shoots were isolated, the remaining calli quickly produced new shoots when transferred to growth regulator-free MS medium. The undifferentiated calli only showed shoot-bud regeneration when transferred to MS medium without growth regulators.

The isolation and culture of mesophyll protoplasts from *S. angustifolia* seedlings has been recently reported (Carvalho et al. 1993). The highest yield of viable protoplasts was achieved with an enzymatic mixture containing 1% cellulase RS and 0.05 or 0.1% Pectolyase Y-23 in 0.5 M mannitol. When cultured in a modified MS liquid medium with several NAA and BA combinations, isolated protoplasts underwent sustained division and produced calli. To date, no reports exist on plant regeneration from *Sideritis* protoplasts.

2.6 Plant Regeneration

Regenerated shoots are easily rooted on growth regulator-free MS medium (Fig. 4). The establishment of the in vitro grown plants in soil is achieved as



Fig. 5. In vitro regenerated plants of *S. angustifolia* after transplanting to pots

follows: plantlets are transplanted to 10-cm diameter pots filled with a soil mixture consisting of equal parts of vermiculite and peat moss (Fig. 5). Subsequently, the plants are adapted to growth chamber conditions ($26 \pm 2^\circ\text{C}$ with a 16-h photoperiod of 20 W m^{-2}) with gradual exposure to reduced relative humidity by progressively removing a glass cover over a period of 2–3 weeks. Once acclimatization is accomplished, plants are transferred to the greenhouse (Sánchez-Gras and Segura 1987).

2.7 Effects of Spermidine on Morphogenesis

There is ample evidence to support the involvement of polyamines in several growth and developmental processes in higher plants (Evans and Malmberg 1989; Bagni and Torrigiani 1992; Minocha and Minocha 1995).

Hypocotyls, cotyledons and roots from *S. angustifolia* were cultured on MS medium supplemented with various concentrations of spermidine (Spd), dicyclohexylamine (DCHA, an inhibitor of Spd synthesis), NAA, IAA, and BA (Sánchez-Gras and Segura 1988b). The results showed that Spd alone did not induce morphogenesis in *S. angustifolia*, although it did affect growth induced by NAA, IAA, or BA. The effect of Spd on NAA-induced growth varied with explant type and auxin concentration. An inhibitory effect was produced when NAA concentrations were optimal for callus induction ($10.8\text{ }\mu\text{M}$ in hypocotyls and roots or $27\text{ }\mu\text{M}$ in cotyledons). In contrast, Spd promoted growth in media supplemented with NAA levels that were toxic for callus induction and growth.

Root regeneration also depended on explant type and on NAA and Spd concentrations. In general, this polyamine did not modify the rhizogenesis induced by 0.5 or 5.4 μ M NAA (Table 3). In media supplemented with 10.8 μ M NAA (optimal concentration for root regeneration), Spd reduced rhizogenesis in cotyledons and hypocotyls, while in roots it had a significant promoting effect (Table 3). The most remarkable effect of Spd on rhizogenesis was observed in media supplemented with 27 μ M NAA and 0.01 mM of the polyamine. Under these conditions, Spd reversed the toxic effect on root formation caused by 27 μ M NAA (Table 3). The effect of Spd on growth induced by IAA or BA (data not shown) was similar to those previously stated with NAA, especially in relation to the inhibitory effect of this polyamine on callus growth induced by the optimal IAA or BA concentrations (11.4 and 5.4 μ M, respectively) and the neutralization of toxicity elicited by the highest IAA or BA concentrations (28.5 and 27 μ M, respectively). Likewise, DCHA reduced both callus growth and rhizogenesis on hypocotyls cultured in the presence of 10.8 μ M NAA or 11.4 μ M IAA (data not shown).

Table 3. Effect of NAA and Spd on root induction in root, hypocotyl, and cotyledon explants of *S. angustifolia*. Culture time was 4 weeks. Within each type of explant, values followed by the same letter are not significantly different at the 5% level using Duncan's Multiple Range Test. (Sánchez-Gras and Segura 1988b)

NAA (μ M)	Spd (mM)	Root		Hypocotyl		Cotyledon	
		Rhizogenic explants (%)	No. of roots explant ⁻¹	Rhizogenic explants (%)	No. of roots explant ⁻¹	Rhizogenic explants (%)	No. of roots explant ⁻¹
0.0	0.00	0	0.00 g	0	0.00 h	0	0.00 g
0.0	0.01	0	0.00 g	0	0.00 h	0	0.00 g
0.0	0.10	0	0.00 g	0	0.00 h	0	0.00 g
0.0	1.00	0	0.00 g	0	0.00 h	0	0.00 g
0.5	0.00	55	1.10 fg	17	0.20 g	0	0.00 g
0.5	0.01	40	0.80 g	17	1.20 g	0	0.00 g
0.5	0.10	40	1.00 g	29	0.55 g	5	0.70 def
0.5	1.00	50	1.60 ef	20	0.20 g	5	0.05 g
5.4	0.00	90	8.40 c	89	5.10 cde	15	0.25 f
5.4	0.01	100	11.50 b	85	6.16 bcd	15	0.15 f
5.4	0.10	90	8.30 c	86	6.90 bc	25	0.40 ef
5.4	1.00	100	8.40 c	70	6.40 bcd	25	0.60 ef
10.8	0.00	85	8.70 c	80	7.30 ab	30	1.80 b
10.8	0.01	100	14.00 a	87	9.20 a	25	1.20 cd
10.8	0.10	95	11.70 b	78	5.30 bcd	15	0.15 f
10.8	1.00	95	12.00 b	64	3.50 ef	10	0.30 f
27.0	0.00	50	2.30 ef	29	1.70 fg	31	0.90 de
27.0	0.01	85	9.50 c	70	4.70 de	65	3.90 a
27.0	0.10	75	2.60 de	56	2.20 fg	25	1.50 bc
27.0	1.00	55	3.60 d	58	3.40 fg	10	0.40 ef

The hypocotyl-derived calli obtained in the presence of 0.01 mM Spd and 27 μ M NAA gave rise to pro-embryonal masses and free somatic embryos (Fig. 6) when transferred to liquid medium without growth regulators. These pro-embryonal masses proliferated quickly when placed on growth regulator-free solidified medium and subsequently regenerated both shoots and complete plantlets. Once the developed shoots and plantlets were excised, the remaining calli maintained this regenerative capability in successive subcultures. Since media supplemented only with NAA failed to promote either caulogenesis or embryogenesis, our results suggest that Spd played an active role in the processes leading to the development of organized structures in cultured tissues of *S. angustifolia*. These results support other works that established a significant correlation between polyamines and somatic embryo development (see Minocha and Minocha 1995).

Results commented above, in conjunction with our success in regenerating plants from isolated cells of *S. angustifolia* (see Sect. 2.5) prompted a new investigation to study the role of Spd in the control of cell proliferation and differentiation. In this experiment, single cells from hypocotyl-derived callus were cultured in either solidified or liquid MS medium supplemented with various concentrations of Spd, NAA, BA, and DCHA (Sánchez-Gras and Segura 1990). For solid cultures, single cell suspensions (10^4 cells/ml) were cultured for 30 days under dark conditions. For liquid cultures, cell suspensions were incubated under a 16-h photoperiod on a horizontal gyratory shaker (100 rpm).

Spd affected growth induced by NAA or BA, although it did not replace the exogenous growth-regulator requirements of *S. angustifolia* cells (Tables 4, 5), which seem to be necessary for cell division and callus formation. This

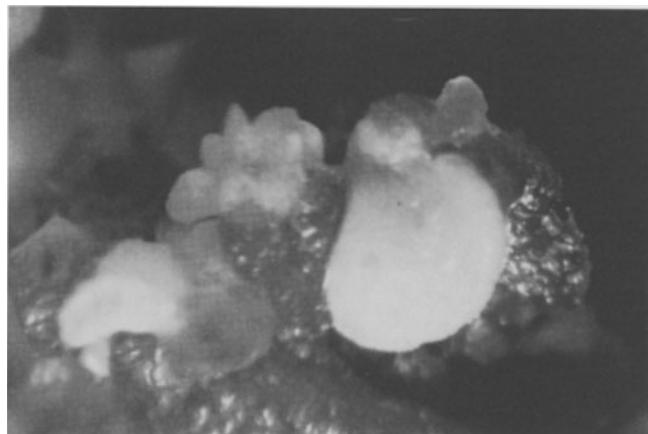


Fig. 6. Hypocotyl-derived callus of *S. angustifolia* showing pro-embryonal masses and free somatic embryos

polyamine increased the plating efficiency of cells cultured in the presence of NAA concentrations ranging from 0.05 to 5.4 μ M (Table 4) or BA concentrations in a range of 0.4–8.8 μ M (data not shown). In contrast, Spd reduced growth induced by 10.8 μ M NAA, optimal auxin concentration for callus induction. These results corroborate those previously obtained with cultured primary explants of this species and suggest an important role of this amine as a modulator of hormone action. Likewise, DCHA reduced the number of cell-derived calli obtained with 5.4 μ M NAA (Table 5), which also confirms the involvement of Spd in the regulation of growth in this species.

To induce caulogenesis, calli grown from plated cells were transferred to various regeneration media (Table 6). Bud differentiation from these calli was mainly affected by the medium initially used for cell culture. Thus, out of treatments tested for cell cultures, only those shown in Table 6 led to calli, which in subsequent subcultures gave rise to shoot-buds. However, some of these calli failed to produce well-developed shoots and lost their caulogenic capacity over the culture period. Since shoot-bud regeneration was only observed in calli initially grown in the presence of Spd, our results seem to corroborate the relationship established by some authors (Torrigiani et al.

Table 4. Effect of NAA and Spd on cell proliferation and callus formation in plating cultures of isolated cells from hypocotyl-derived callus of *S. angustifolia*. Cells were cultured at 10^4 cells/ml for 4 weeks. (Sánchez-Gras et al. 1990)

NAA (μ M)	Spd (mM)	No. of calli per dish
0	0	0 i
0	0.001	0 i
0	0.010	1.6 i
0	0.100	0 i
0.05	0	0.3 i
0.05	0.001	1.6 i
0.05	0.010	2.2 i
0.05	0.100	26.6 h
0.54	0	4.6 i
0.54	0.001	6.0 i
0.54	0.010	6.9 i
0.54	0.100	104.1 c
5.40	0	65.1 f
5.40	0.001	77.4 e
5.40	0.010	131.9 b
5.40	0.100	90.3 d
10.80	0	219.8 a
10.80	0.001	95.4 cd
10.80	0.010	41.2 g
10.80	0.100	23.4 h

Values followed by the same letter are not significantly different using Tukey's test.

Table 5. Effect of different medium supplements on cell growth in liquid and plating cultures of isolated cells from hypocotyl-derived callus of *S. angustifolia*. Cells were cultured at 10⁴ cells/ml for 10 days (liquid test) or 4 weeks (plate test). (Sánchez-Gras et al. 1990)

Medium supplement	Liquid test		Plate test No. calli/dish
	PCV (ml)	No. cell (10 ⁴)	
5.40 µM NAA	0.099 (IV)	1.00 (IV)	—
0.01 mM Spd	0.218 b	1.66 b	65.1 b
5.40 µM NAA/0.01 mM Spd	0.098 e	0.93 c	1.6 e
5.40 µM NAA/1 mM DCHA	0.280 a	2.45 a	131.9 a
5.40 µM NAA/10 mM DCHA	0.152 c	1.47 b	50.6 c
5.40 µM NAA/10 mM DCHA	0.123 d	1.00 c	36.7 d

Within each column, values followed by the same letter are not significantly different using Tukey's test.

IV, initial values.

PCV, packed cell volume.

Table 6. Comparative caulogenic responses of calli derived from isolated cells of *S. angustifolia*. Only those data from cell-derived calli that showed regenerative capacity are shown. (Sánchez-Gras et al. 1990)

Cell culture medium	Bud induction medium			Caulogenesis (after 30 days)		Elongation medium		Caulogenesis (after 30 days)		
	Spd (mM)	NAA (µM)	BA (µM)	NAA (µM)	BA (µM)	Calli with buds (%)	NAA (µM)	BA (µM)	Calli with buds (%)	No. shoots ≥1 cm
0.10	0.05	0.00	0.00	0.00	0	0	0.00	0.00	0	0
						0.05	0.44	0	0	0
				4.44	35	0.00	0.00	65	24	
	0.54	0.00	0.54	4.44	11	0.05	0.44	20	0	
						0.00	0.00	60	23	
						0.05	0.44	15	0	
	0.10	0.54	0.00	0.00	0	0	0.00	0.00	0	0
						0.05	0.44	0	0	
				4.44	25	0.00	0.00	0	0	
0.01	5.40	0.00	0.00	0.00	0	0.05	0.44	0	0	
						0.00	0.00	0	0	
						0.05	0.44	0	0	
	0.01	4.40	0.00	0.00	67	0.00	0.00	87	5	
						0.05	0.44	75	1	
				4.44	67	0.00	0.00	100	8	
0.01	0.00	4.40	0.54	4.44	100	0.05	0.44	100	0	
						0.00	0.00	85	29	
						0.05	0.44	75	4	

1987; Biondi et al. 1988; Martin-Tanguy et al. 1988; Burtin et al. 1989) between endogenous polyamine levels and bud differentiation.

3 Summary

Micropropagation of *S. angustifolia* Lag. was achieved through organogenesis and somatic embryogenesis. Shoot regeneration was induced in hypocotyl, root, cotyledon, and leaf explants. The highest shoot-bud regeneration rate was obtained in hypocotyls cultured in MS medium supplemented with $5.4\text{ }\mu\text{M}$ NAA and $4.44\text{ }\mu\text{M}$ BA. The influence of explant age and illumination conditions was also tested in leaf explants, obtaining the best caulogenic response in young leaves preincubated in darkness. Conditions required for plant regeneration from single cells, isolated from hypocotyl-derived callus, were also determined. Maximal shoot regeneration rates occurred when cell-derived calli, grown in the presence of $0.5\text{ }\mu\text{M}$ NAA, $8.8\text{ }\mu\text{M}$ BA, and 100 mg/l CH, were subcultured in the same medium without CH. These calli maintained their regenerative capacity along several subcultures in MS medium without growth regulators. Regenerated shoots developed roots when transferred to medium without growth regulators. Rooted plantlets were successfully transplanted into soil. Pro-embryonal masses and free somatic embryos were observed when hypocotyl-derived calli, induced with 0.01 mM spermidine and $27\text{ }\mu\text{M}$ NAA, were transferred to liquid medium without growth regulators. These somatic embryos developed into plantlets after subculturing on solidified medium without growth regulators.

Micropropagation of *S. foetens* was carried out culturing nodal sections with two axillary buds. The best shoot proliferation rate was achieved in explants cultured in MS medium supplemented with $0.22\text{ }\mu\text{M}$ BA. Rooting of regenerated shoots was done *in vivo*.

4 Protocol for *S. angustifolia* Cultures

4.1 Micropropagation

1. Hypocotyl explants from 30-day-old seedlings are cultured on agar-solidified medium containing $5.40\text{ }\mu\text{M}$ NAA and $4.44\text{ }\mu\text{M}$ BA for bud induction. Buds appear after 2–3 weeks of incubation at $26 \pm 2^\circ\text{C}$ under a 16-h photoperiod.
2. Cultures bearing buds are transferred to MS medium without growth regulators for shoot development.
3. The obtained shoots are transplanted to rooting medium (growth regulator-free MS medium). Rooted plantlets are subsequently acclimatized to greenhouse conditions after gradual hardening off.

4.2 Single Cell Culture

1. Hypocotyl explants from 30-day-old seedlings are cultured on MS medium supplemented with $0.5\mu\text{M}$ NAA and $8.8\mu\text{M}$ BA for callus proliferation. Cultures are incubated for 30–40 days at $26 \pm 2^\circ\text{C}$ under a 16-h photoperiod.
2. The obtained calli are transferred to liquid MS medium and agitated for 12 h (130 rpm) at $26 \pm 2^\circ\text{C}$. The resulting cell suspension is filtered through a stainless steel sieve ($90\mu\text{M}$) and centrifuged (1500 rpm for 6 min).
3. Isolated cells are cultured, at 10^4 cells/ml, on solidified MS medium supplemented with $0.5\mu\text{M}$ NAA, $8.8\mu\text{M}$ BA and 100mg/l CH, and kept in darkness at $27 \pm 1^\circ\text{C}$ for 30 days.
4. To induce organogenesis, cell-derived calli are transferred to MS medium supplemented with $0.5\mu\text{M}$ NAA and $8.8\mu\text{M}$ BA. Buds appear after 4 weeks of incubation at $26 \pm 2^\circ\text{C}$ under a 16-h photoperiod.
5. For shoot development, rooting and acclimatization proceed as indicated in micropagation protocol.

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II.4 Microppropagation of *Dictamnus albus* L. (Gas Plant)

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1 General Account

1.1 Botany, Distribution, and Importance

Gas plant (*Dictamnus albus* L., family Rutaceae) is an attractive, long-lived herbaceous perennial that has been cultivated for centuries. Written accounts of its medicinal properties appear as early as 1 A.D. in Dioscorides' *Materia Medica* and continue in herbals of the seventeenth century. John Gerard documents gas plant growing in his garden in 1597 (Gerard 1597). It is native to Europe and Asia (Everett 1981). Once established, plants continue to flourish for decades, eventually creating a fantastic show of flowering spikes up to 2 ft. tall. Because of the fleshy root system, these plants do not transplant well after they are established. The extensive root system also allows the plant to tolerate dry conditions in the garden. The foliage is a rich green, resembling ash leaves and is attractive even after the flowers fade. The flowers appear in an upright raceme (spike) and are white to purple, often with prominent streaks of red on the petals (Fig. 1). Individual flowers have five petals and prominent anthers. All flower and fruit parts contain glandular trichomes (Fig. 2).

The common name, gas plant, originates from the practice of igniting the volatile exudates from the glandular trichomes on the flowers or fruit. On still evenings, this produces a brief flame without apparent harm to the plant (Still 1988). The first Latin name ascribed to gas plant was *Tragium*. This is a variation of "Tragos," the Greek word for goat, and was used because the pods supposedly smell like a goat. Why this name was abandoned for *Dictam* and finally *Dictamnus* is not clear. In an attempt to avoid confusion with Dittany (*Origanum*), herbalists in the seventeenth century usually used the name *Fraxinella* (small ash). Although this name was very descriptive of the gas plant, Carl Linnaeus chose to describe gas plant as *Dictamnus albus*. This is the accepted name today, but many references still list *Fraxinella* or *Dictamnus fraxinella* as synonymous because *Fraxinella* was the most commonly used name for this plant in the early 1900s.

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Fig. 1. Flower spike in gas plant. (Geneve 1996)**Fig. 2.** Close-up of a single flower of gas plant showing prominent glandular trichomes

Available horticultural selections of gas plant include white and pink flowering forms. It is accepted that *D. albus* is a white flowering form (although all shades of pink to purple are possible from seed). The two most commonly cited pink or purple forms are 'Purpureus' and 'Rubra'. However, there is considerable confusion concerning the integrity of these cultivars. Since flower color comes relatively true from seed, these cultivars have been

maintained as variable seed populations or vegetative propagation of selected seedlings. Seed propagated selections like 'Rubra' may best be considered a botanical variety rather than a uniform cultivar. 'Albiflorus' is a white selection of gas plant. In addition, *D. albus* var. *Caucasicus* is a botanical variety of gas plant that is larger and more robust than the species. However, this plant may be synonymous with the cultivar 'Purpureus'.

The flammable compounds produced by gas plant are most likely monoterpenes. The citrus odor emitted by gas plant is probably the monoterpenes, limonene. The large amounts of these compounds and the low flash point of monoterpenes helps explain the unique flammable nature shown by gas plant flowers and seed pods. These compounds have been shown in other species to deter insect feeding. In this regard, gas plant is remarkably pest-free.

The complex mix of secondary plant products that provides gas plant with both its flammable nature and citrus aroma also includes some noxious chemicals. The major alkaloid in gas plant is dictamnine (Souleles 1989). This is a furoquinoline alkaloid and has been shown to be mutagenic. Dictamnine, along with a second compound, bergapten, is found in the aboveground parts of gas plant and are responsible for a serious contact dermatitis (Cummer and Dexter 1937; Hipkin 1991). Affected areas blister severely. This type of contact dermatitis is called phytophotodermatitis producing skin irritation when exposed to ultraviolet A radiation.

1.2 Conventional Propagation and Need for Micropropagation

Gas plant has limited availability in commercial nurseries because it is difficult to propagate. It has been propagated by seed, division and root cuttings (Everett 1981; Still 1988; Armitage 1989); however, each of these methods presents problems for the commercial propagator.

Seed would be the preferred method for the propagation of gas plant by commercial growers. This method is inexpensive and flower color comes relatively true from seed. However, seeds have a complex dormancy that does not appear to be consistent across seed lots or from season to season in seeds collected from the same source (Jones 1993). The Association of Official Seed Analysts rules for testing seed (1993) recommends prechilling gas plant seeds for 45 days prior to germination at 20–30°C with light. Unfortunately, this treatment can lead to limited germination in some seed lots (Pinnel et al. 1985).

Jelitto and Schacht (1985) suggest that seeds should be sown directly after ripening for best germination. This type of seed behavior has been observed in other members of the Rutaceae including *Zanthoxylum*, *Citrus*, and *Ptelea*. In *Zanthoxylum*, it was observed that stored seed developed a strong dormancy (Bonner 1974) and *Citrus* seeds germinate with difficulty after drying (King and Roberts 1980). Jones (1993) collected seed from gas plant at physiological maturity before desiccation drying (56% moisture) and immediately after drying (9.5% moisture). Neither seed lot germinated without chilling stratification and there was no significant difference between germination percentage after 12 weeks of chilling stratification at 5°C. Germination for fresh seed was

37%, while germination for dried was 43%. However, fresh seed reach maximal germination after only 6 weeks of chilling, while dried seed required 12 weeks to reach maximal germination.

Seed quality is an important factor in gas plant germination. Tetrazolium staining for viability (1% solution for 2 h at 5 °C) revealed that as many as 48% of a given seed lot can be nonviable (Jones 1993). This explains some of the poor germination percentages reported for gas plant. However, there are seed lots that stain high for viability with tetrazolium and that germinate at low percentages (<10%) after 12 weeks of stratification.

The evidence for seed germination in gas plant indicates that complex dormancy and seed quality are major factors influencing germination. Chilling stratification at 1 or 5 °C for 12 weeks appears to be an appropriate pretreatment for gas plant seeds, but after 2 years of research with five different seed lots, germination percentages never exceed 50% (Jones 1993). Seed germination will remain a viable propagation option for growers, but results suggest that percentages will be low and be inconsistent between seasons and seed lots.

Root cuttings have been used to propagate cultivars of gas plant (Armitage 1989). This is a relatively simple form of propagation, where root pieces are collected from dormant gas plants. Root pieces approximately 5–7 cm in length are planted in flats containing sand (or other suitable medium) with the proximal end of the root just below the surface of the medium. Flats are placed in a cool greenhouse and new shoot growth appears in the spring. The major disadvantage to root cuttings is that they are laborious to collect in quantity. This is an added problem for gas plant because large stock plants cannot be used because they do not recover well after being disturbed. Despite these difficulties, root cuttings from young container-grown plants are being used for limited clonal production.

The availability of gas plant in the nursery trade has been limited because it is difficult to propagate economically. Success with seed propagation has been variable and unreliable. Gas plant is an inherently variable species and there is a need to select superior plants of this species for clonal propagation and maintenance of true-to-type cultivars. Gas plants can be propagated from seed, but the seedlings are variable for a number of plant characteristics. Seed propagation is difficult because of a complex seed dormancy. Micropagation would provide an alternative for the rapid propagation of gas plants. A protocol for the rapid multiplication of gas plant by micropagation consistent with techniques used to propagate other herbaceous perennials through tissue culture could make gas plant more widely available and be a vehicle for the vegetative selection of superior plants.

2 Review of Tissue Culture/Micropagation

Jones (1993) demonstrated that embryos can be isolated from the seed coat and cultured in vitro on half-strength MS salts medium (Murashige and Skoog 1962) supplemented with 2% sucrose. Embryo growth in culture was seed lot-dependent ranging from 2 to 100%. The authors suggested that excised em-

bryo germination could be a good indicator of the dormancy condition in a seed lot. Seed lots with high in vitro embryo growth also germinated well after chilling stratification. Seed lots with low in vitro embryo growth failed to germinate (<10%) after 12 weeks of chilling stratification even though the stratified embryos stained positive for viability using tetrazolium.

Root pieces from seedlings grown in vitro have been isolated and grown in culture (Jones 1993). It might be expected that root pieces would readily form shoots in culture because ex vitro root cuttings have the potential to regenerate shoots. However, root pieces grown on MS or White's medium supplemented with various concentrations of auxin grow very slowly in culture and show very little lateral root initiation. Similarly, gas plants in the nursery transplant poorly because of limited root regeneration. In vitro root cultures do not appear to be a viable option for micropropagation of gas plant, but root cultures may offer a good model system to study recalcitrant root regeneration which can be a significant problem in the survival of ornamental plants in the landscape.

Shoot formation in vitro has been reported by Zilis et al. (1979) in an overview of herbaceous perennials micropropagated by Walters' Gardens (Michigan, USA). Few specific details on culture medium and environment were provided in this study, but the authors indicated that shoot formation was possible from organized shoot explants of gas plant. However, attempts to root microshoots of gas plant in vitro were not successful with rooting and acclimatization at less than 50%. Recently we (Jones et al. 1994) have published a successful protocol for micropropagation of gas plant, and the results are summarized here.

2.1 Establishment of Cultures

Emerging shoots from greenhouse-grown plants of flowering age (3 years old) were used as initial explants. Stems were disinfected in a 20-s quick dip of 70% ethanol followed by 10-min agitation in 0.5% sodium hypochlorite (10% Clorox) plus 0.1% Alconox detergent. Stems were cut into two-node pieces and placed horizontally in autoclaved Magenta jars (Sigma, St. Louis, Missouri), containing MS medium with macro- and micronutrients, solidified with 0.7% Difco Bacto-agar and supplemented with 2% sucrose and 1 μ M benzyladenine (BA). Cultures were placed under 40-W cool white fluorescent bulbs at approximately $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR with a 16-h photoperiod and a temperature of $23 \pm 2^\circ\text{C}$. Subculturing was performed every 6 weeks. Dose response studies were conducted after enough shoots were established on this medium.

2.2 Shoot Multiplication

Four-node in vitro developed shoots were placed in a horizontal or vertical orientation in Magenta jars containing 40 ml of MS medium supplemented

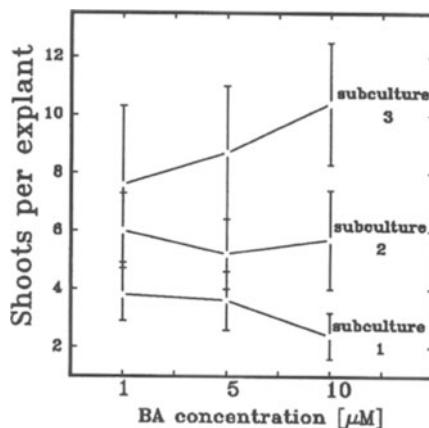


Fig. 3. Effect of benzyladenine and subculturing on in vitro shoot formation in gas plant. (Jones 1993)

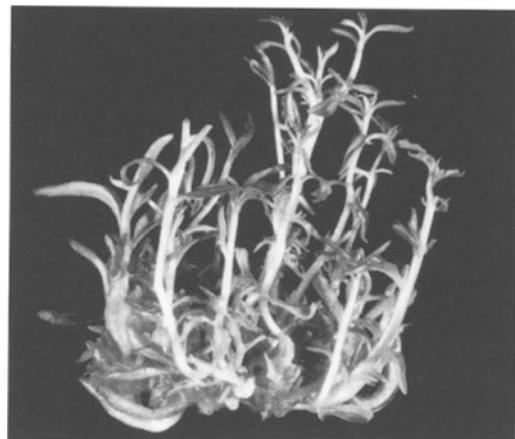


Fig. 4. Shoot formation in stabilized gas plant cultured after 6 weeks on 1 μ M benzyladenine. (Jones et al. 1994)

with 1, 5, or 10 μ M BA. After the shoots had been removed and counted, the original explants were subcultured on fresh medium at the same BA concentration. There were 32 explants per treatment.

Explants readily formed shoots in culture (Figs. 3, 4). The number of shoots per explant increased for each subculture (Fig. 3). This may be the result of stabilization of the cultures or increased adventitious bud formation. During each subculture, there was little difference in the number of shoots induced by any BA concentration. However, by the third subculture there was a trend for more but shorter shoots produced in the 10 μ M BA cultures. After six subcultures, explants produced on a medium with 1 μ M BA had 70.5% of

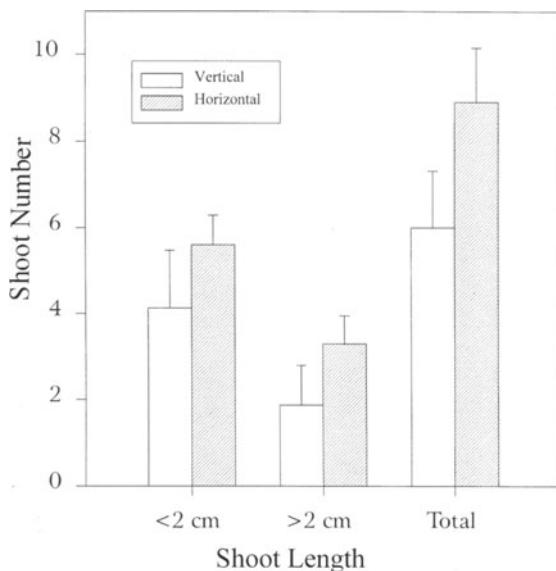


Fig. 5. Shoot formation in explants treated with 5 µM benzyladenine using a horizontal or vertical orientation. (Jones 1993)

shoots longer than 2 cm. This contrasted to less than 50% for 5 and 10 µM BA cultures. The number of shoots per culture was greater in explants placed horizontally on the medium compared to those with a vertical orientation (Fig. 5). This resulted in a 35% increase over the three subcultures used for evaluation.

2.3 Root Formation and Acclimatization

All microcuttings used were >2 cm long. Cuttings were evaluated for root formation in vitro or under ex vitro rooting conditions. In vitro root formation involved placing single microcuttings in culture tubes containing half-strength MS medium supplemented with 0, 10, 50, or 150 µM indolebutyric acid (IBA). Microcuttings were also treated with a 5-s quick dip with IBA at 0, 1000, 5000, or 10000 ppm in 50% ethanol. Treated microcuttings were transferred directly into ex vitro rooting conditions in cell packs filled with a peat-lite greenhouse medium. Cell packs were placed in flats covered with a clear plastic top and enclosed in a clear polyethylene bag. A minimum of 35 microcuttings were used for each treatment. Microcuttings were evaluated for root formation after 6 weeks. Rooted microcuttings were acclimatized over a 3-week period in a growth chamber by gradually reducing the humidity.

Microcuttings rooted less than 20% under in vitro conditions confirming the difficulty this species has with in vitro root formation (Zilis et al. 1979). However, microcuttings rooted between 71 and 86% under ex vitro rooting

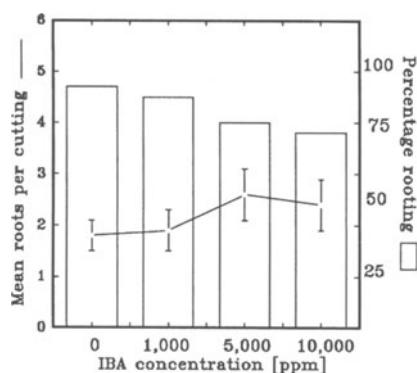


Fig. 6. Root formation in gas plant microcuttings treated with a quick dip of indolebutyric acid (IBA) under ex vitro conditions. (Jones 1993)

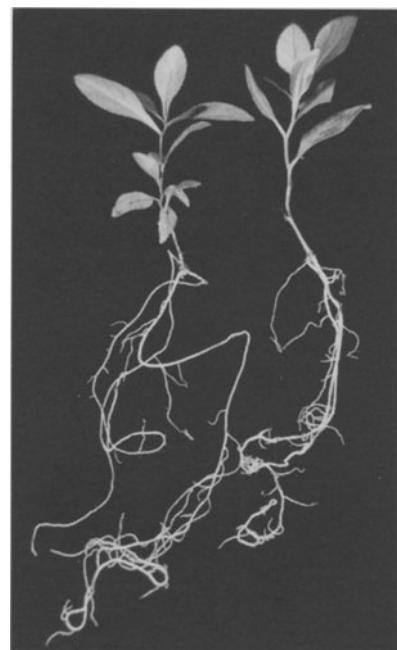


Fig. 7. Root formation in gas plant microcuttings showing tap-rooted nature of plantlets. (Jones 1993)

conditions (Fig. 6). IBA at 5000 and 10000 ppm slightly increased the number of roots per cutting, but decreased the percentage rooting by 13–15%. Two roots per cutting is a relatively low number for most micropagated crops; however, these roots assumed the role of a primary tap root (Fig. 7), which is typical of gas plant, and plants developed an extensive root system after 1 year



Fig. 8. Acclimatized micropropagated plants of gas plant after 1 year's growth in the greenhouse. (Jones et al. 1994)

(Fig. 8). Rooted cuttings acclimatized at more than 80% and flowered normally in the greenhouse.

3 Protocol

Gas plant has been micropropagated successfully following procedures consistent with other perennials propagated in vitro. The best shoot multiplication was found for horizontally placed explants on MS medium for stabilized cultures containing 1 or $5\mu\text{M}$ BA. After 6 weeks, microcuttings have rooted and are ready to be acclimatized. Microcuttings root best after being placed in a peat-lite medium and maintained at near 100% humidity. Untreated microcuttings root as well as IBA-treated cuttings. Rooted cuttings can be acclimatized to ambient conditions by gradually reducing humidity over a 3-week period and plants appear to be growing normally in the greenhouse.

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II.5 Microppropagation of *Simmondsia chinensis* (Jojoba)

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1 Introduction

1.1 Botany, Distribution, and Importance

Jojoba [*Simmondsia chinensis* (Link) Schneider], a new oil-seed crop, is a perennial evergreen shrub native to the Sonora Desert of the south-western USA and northern Mexico (Fig. 1). There are male and female plants whose populations are found in diverse climatic, geographic, and edaphic conditions, at altitudes from sea level to about 1200m, usually on coarse, sandy, or gravelly soils with good drainage. Native jojoba populations can be found in areas receiving an annual precipitation of 80–450 mm and having temperatures ranging from –9 to 50 °C (Gentry 1958). The plant is drought-resistant and to some extent also salt-resistant (Yermanos et al. 1967; Benzioni and Dunstone 1986; Benzioni et al. 1992; Mills and Benzioni 1992).

The dry seeds of jojoba contain about 50% lipids in the form of simple wax esters that have properties similar to those of sperm whale oil. The wax esters have an average chain length of 42 carbons and are made up of mono-unsaturated fatty acids and alcohols of 20–24 carbons (Miwa 1980; US National Research Council 1985). The wax, commonly known as jojoba oil, and its derivatives have potential in a wide range of applications in cosmetics, pharmaceuticals, lubricants, gear additives, extenders, anti-foaming agents, and in the wax and polish industries. Today, jojoba oil is used mainly in the cosmetics industry. It is likely that as worldwide production increases beyond the capacity of the cosmetics industry, and the price thus decreases, jojoba oil will penetrate into other larger markets.

The maximal yields of commercial fields established from seeds under the best agromanagement and climatic conditions is about 1–2 ton/ha. Plantations of selected clonal material may reach 3–4 ton/ha (Benzioni 1995). Increases in the yield would enable a reduction in oil prices to 6–8\$/kg, a price that is too high for the lubrication industry but low enough for other special uses in industry (Benzioni 1995). The process of selecting high-yielding clones from superior plants for specific sets of environmental conditions is still in its infancy and must be continued if the industry is to develop further.

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Fig. 1. Shoot-bearing fruits of a female jojoba shrub

Jojoba was introduced to many countries during the late 1970s and early 1980s. During that period, seeds constituted the propagation material. Many of the plantations established from seed did not yield well, and the need for plant material from selected genotypes adapted to specific climatic and edaphic conditions was recognized. Today, commercial plantations from seedlings still exist, but new plantations are grown from vegetatively propagated material. Currently, the main jojoba oil producers are Israel, the USA, Argentina, Australia, and Mexico.

1.2 Conventional Propagation

Jojoba may be propagated either through seed or vegetatively. Seeding is carried out directly into the field at depths of 2–5 cm in wet, well-drained soil. Transplanting of seedlings raised in the nursery is also possible. The latter method has been used in the past (Yermanos 1978), and today it is used mainly for gap filling. Variation in seeded plantations is huge due to the fact that jojoba is a dioecious, cross-pollinating species. A seeded plantation of jojoba has genetic heterogeneity, which is expressed in phenological, morphological, anatomical, physiological, and production characteristics (Yermanos 1977; Purcell and Purcell 1988); for example, seed yield from a 7-year-old plantation varied from 0 to 2200 g/plant (Purcell and Purcell 1988).

A seeded plantation contains 50% male plants, while 8–10% are sufficient for pollination. As male plants are not only unproductive but also suppress the development of adjacent females, rouging of the superfluous males is imperative.

Yield improvement can be obtained through a recurrent selection program by increasing the frequency of high-yielding plants in each generation (Purcell and Purcell 1988; Naqvi et al. 1990). Since significant yields are obtained only from the fourth year, and yields have to be followed for at least an additional 4 years, this strategy becomes a very long-term process (about 8 years for each step). Direct selection based on identifying potentially promising genotypes and their testing after vegetative propagation shortens improvement procedures and is more feasible than breeding programs (Purcell and Purcell 1988). Vegetative propagation will enable the establishment of plantations with the desired proportion of male to female plants of preselected superior clones. The primary criterion for selection is high yields. For example, a group of some selected clones in Mexico yielded 160% more than seeded plants of the same age in the same field (Ramonet and Morales 1984). It should be noted that variability in yield still exists within a population of plants of the same clone but is lower than in a population of seeded plants. Plants of a particular clone are uniform in other traits, such as canopy size and flowering date (Ramonet and Morales 1984). From a population of preselected plants, further selection based on a variety of criteria must be continued; for example, high or low chilling requirements may enable allocation of genotypes to different climatic conditions. Frost resistance, pest and disease resistance, salt resistance, appropriate shape, earliness of coming into production, oil content and constituents, and seed size are some of the criteria already being used in the selection of genotypes for new plantations. Another advantage of vegetatively propagated plants is that they mature reproductively sooner than seeded plants (Ramonet and Morales 1984).

Among the methods used for conventional asexual propagation are air-layering (Alcaraz 1980; Alcaraz and Ayala-Rocha 1982) and grafting (Mirov 1973). A rooting percentage of 90% of air-layered stems has been obtained with 20mM indole-3-butyric acid (IBA) and 20mM naphthaleneacetic acid (NAA) (Alcaraz 1980 and Ayala-Rocha 1982). Neither of these methods is very successful: both are labor-intensive, and grafting is usually followed by a burst of branches from the base of the grafted plant, which makes the latter method unreliable. The most practical, and today the only, commercial method is rooting of cuttings harvested from selected plants in a mother plantation. The most comprehensive review of this system for propagating jojoba was prepared by Palzkill (1988).

In general, cuttings of jojoba root within 3–5 weeks when substrate temperatures are kept at 25–30°C and shoots are misted. In this way, rapid multiplication of certain clones can be achieved. In a study reported from Arizona, propagation was accomplished by splitting the bottom of cuttings containing six to eight nodes with a razor blade, dipping them in an IBA solution and placing them in a rooting medium of equal parts of perlite, peat and vermiculite (Brown and Cambell 1984). Cuttings were grown in a fogged greenhouse. Wounding the cutting, which seemed to create callus and facilitate root initiation, in combination with IBA application, was found to be the most effective means of rooting the cuttings. The percentage of rooting ranged

from 0 to 70% (Brown and Cambell 1984) or 0 to 85% (Abramovich et al. 1985), depending on the genotype and treatment.

In Israel, a methodology for rooting cuttings was recently established by the nursery of Kibbutz Hazerim. Three-node cuttings were kept in a refrigerator for 48 h, dipped in rooting powder containing IBA, and then placed in a perlite:peat:foamed polystyrene (1:1:1) substrate in peat pots (known as Jiffys). The cuttings were held in temperature-controlled beds in a temperature-controlled greenhouse. Mist irrigation was applied for 10 s every 10 min. Rooted cuttings were transferred to final pots after about 4–5 weeks and gradually hardened by transferring to unheated tables, and then to a net house (30% shade). Plants were ready for transplantation to the field after 3–6 months. Rooting percentage ranged from 15 to 95%, depending on the clone and the season, with an annual mean of 65%. A high rooting percentage was obtained in winter and spring (Benzioni 1995). Low and Hackette (1981) found, however, that the best rooting was obtained in summer. Variation of rooting capability between different mother plants was also reported (Low and Hackette 1981; Palzkill 1988).

At present, rooting of cuttings is the commercially preferred method of propagation. In vitro propagation is an alternative method, which offers some important advantages: (1) in terms of mass propagation, the technique is not limited by the number of explants; and (2) pathogen-free plants can be produced. The application of this type of technique depends, however, on factors such as production costs and the demand for propagated plant material.

2 In Vitro Culture Studies

In addition to micropropagation studies have been conducted on zygotic and somatic embryos (embryogenesis), callus (organogenesis), and protoplasts. Wang and Janick (1986a) showed that immature zygotic embryos cultured in vitro could accumulate up to 39% wax on a dry weight basis. Growth and wax biosynthesis were inferior in liquid culture.

Lee and Thomas (1985) and Wang and Janick (1986b,c) established a system for the induction of somatic jojoba embryos from immature zygotic embryos for a number of purposes: (1) as a screening tool for clones with a high oil content since one embryo can be used for the assay and the remainder for propagation; and (2) as a source of liquid wax. Explants smaller than 2 mm turned brown and died, while larger ones continued to develop without the further induction of somatic embryos (Lee and Thomas 1985). In contrast, Unique (1990) reported that the largest embryos (7–10 mm) gave the best embryogenesis rate of up to 80% when cut transversally every 2–3 mm. The maximal fresh weight gain was obtained on a medium containing 3% sucrose, while the maximal oil content was found at 40% sucrose (Lee and Thomas 1985).

Different organs of jojoba were tested for their callogenetic and organogenic capacities (Unique 1990). Upon exposure to 10 μ M 2,4-dichlorophenoxyacetic acid (2,4-D), sepals and ovaries (0.5 mm wide) of dormant flowers produced no callus, while stigmata produced little callus, and immature ovules with 4–6-mm wide ovaries developed massive green callus. Callus developed from 1.5- to 2-mm wide ovaries regenerated into meristematic structures, shoot apices, and foliar primordia.

Protoplasts for cytological and lipid biosynthesis research were isolated from tissue-cultured leaves or callus with cellulase, Onozuka RS and Pectolyase Y23 (Cantrel et al. 1991). Up to 5×10^6 protoplasts were isolated from 1 g fresh weight of both tissues, with a viability of 80%.

3 Micropagation

Literature on the in vitro propagation of jojoba is rather sparse (Rost and Hinchee 1980; Birnbaum et al. 1984; Lee 1988; Chaturvedi and Sharma 1989), and only two groups of investigators have reported the entire cycle from tissue culture establishment to acclimatization (Birnbaum et al. 1984; Chaturvedi and Sharma 1989).

At the Institutes for Applied Research of the Ben-Gurion University of the Negev, Israel, micropropagation of jojoba is performed in five stages: (1) shoot culture establishment; (2) proliferation; (3) shoot elongation; (4) rooting (induction and growth); and (5) acclimatization. A pretreatment stage was found to be redundant, and it was possible to use explants directly from active growing stems from the field, in contrast to the situation for many other woody species (Litz and Jaiswal 1991).

3.1 Establishment of Aseptic Cultures

Our group has established 30 female and 5 male clones of jojoba, mostly from explants taken from superior shrubs selected in Israel. Axillary buds or a mixture of axillary and apical buds were used. We established clones from a small portion of explants – as little as eight – and reach up to 144 propagules with an average of 48 propagules per clone after 6 months (Table 1). Establishment success of shoot cultures was 100% when explants were taken from active growing stems, while explants from dormant plants were difficult to establish.

The following procedure was used for culture initiation: young apical parts of new branches (5–10 cm in length) were cut and rinsed in tap water containing detergent for removal of dust and spores. Branches were then cut into nodal or apical bud segments and disinfected with 1% sodium hypochlorite and 0.05% Tween 20 for 10 min. The segments were washed three times with sterile distilled water, and the buds were excised with less than 1 mm (thick-

Table 1. Survival and growth of original explants and propagule production in culture. Values are means and ranges for 31 clones

	Months after culture initiation						
	0	1	2	3	4	6	9
No. of original explants							
Mean	40	16	8	4	3	nr	nr
Range	8-86	2-56	1-23	1-15	1-13	nr	nr
No. of growing explants							
Mean	0	0.1	3	3	3	nr	nr
Range		0-2	0-11	0-8	0-6	nr	nr
No. of propagules							
Mean	0	0.2	6	8	22	48	190
Range		0-5	0-24	0-34	0-72	1-144	2-1107

nr, Not recorded.

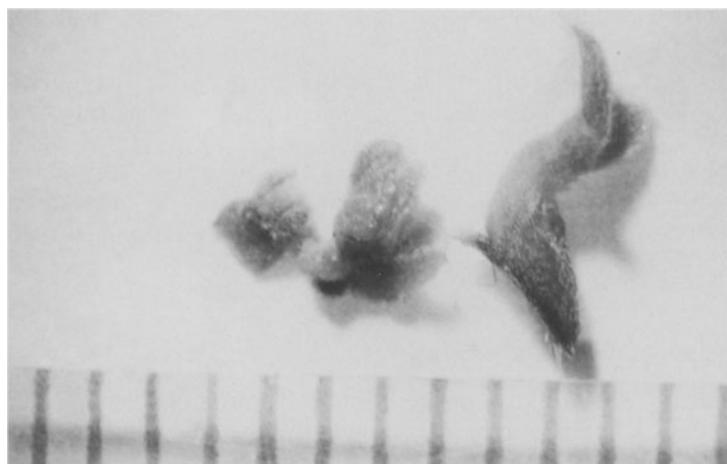


Fig. 2. Development of an apical bud explant. *Left* An excised apical bud; *middle*, initial growth after about a week; *right* elongation after about 2 weeks. Scale is in mm

ness) of underlying shoot tissue attached (Fig. 2). Finally, the excised tissue was cultured on a modified MS (Murashige and Skoog 1962) proliferation medium (Table 2). Excision of the bud leaving only a small section of the underlying shoot tissue enabled us to control massive contamination, mainly by fungi, and also helped to control endogenous bacteria. It is important to distinguish between reproductive and vegetative buds which develop side-by-side in the same leaf axil and are similar in size. While the female reproductive bud resembles a ridge with two peaks and the male is globose, the vegetative

Table 2. Modified MS media used for micropropagating jojoba

Constituent	Media (amounts in mg l ⁻¹) ^a			
	Proliferation	Shoot elongation	Root induction	Root growth
NH ₄ NO ₃	1650	1650	—	825
KNO ₃	1900	1900	—	950
CaCl ₂ ·2H ₂ O	440	440	—	220
KH ₂ PO ₄	170	170	—	85
H ₃ BO ₃	6.2	6.2	—	3.1
MgSO ₄ ·7H ₂ O	370	370	—	185
MnSO ₄ ·H ₂ O	16.9	16.9	—	8.5
ZnSO ₄ ·7H ₂ O	8.6	8.6	—	4.3
CuSO ₄ ·5H ₂ O	0.025	0.025	—	0.0125
CoCl ₂ ·6H ₂ O	0.025	0.025	—	0.0125
KI	0.83	0.83	—	0.42
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	—	0.125
FeSO ₄ ·7H ₂ O	27.8	27.8	—	13.9
Na ₂ EDTA·2H ₂ O	37.3	37.3	—	18.7
Thiamine HCl	0.4	0.4	—	0.4
Nicotinic acid	0.5	—	—	—
Pyridoxine HCl	0.5	—	—	—
Inositol	100	—	—	—
Glycine	—	—	—	—
Adenine sulphate	80	80	—	80
Charcoal	—	5000	—	5000
Sucrose	30000	30000	30000	20000
2iP	30	—	—	—
IAA	0.3	—	—	—
IBA	—	—	120	—
BA	—	—	1.35	—

^aThe pH of all types of medium was 5.8.

bud is shaped like a peaked dome. Planting both types of buds connected together hampered the development of the vegetative bud.

The number of the initial cultured explants dropped significantly in the first 3 months (Table 1): 20% of the decrease was due to fungal contamination, but the main reason was death of the buds. From a mean starting number of 40 explants per clone, three survived after 4 months; all of them developed and could be subdivided into apical and nodal segments. The frequency of bud growth was found to depend on the size of the underlying shoot tissue: if too small, there was callusing of the bud, and if too big, there was no growth. In both cases the buds eventually died. Subdivision took place when explants consisted of two nodes in addition to the apical bud (Fig. 3). Two months after culture initiation, a mean of three buds per clone developed, yielding after a further 7 months an average of 190 propagules per clone (63 per explant). Only propagules originating from one explant were kept for further multiplication, and the rest were discarded.

Fig. 3. Apical segment after 4 weeks of growth now ready for subdivision (shoot length is 20mm)



Establishment of jojoba shoot cultures by Chaturvedi and Sharma (1989) involved a more complicated disinfection process. They treated single-node stem segments first in running water for 30min, followed by a 5% Teepol solution for 20min, and 95% ethanol for 5s, 0.1% HgCl_2 for 10min, and finally Cl_2 -saturated water for 5min. Despite this rigorous treatment only 10% contamination-free cultures were obtained. Rost and Hinchee (1980) disinfected 1-cm-long stems or 0.5- to 1.0-mm apices with 5.25% NaOCl for 20min, followed by three rinses with distilled water. The percentage of contamination or death of explants was not reported.

We also attempted to establish cultures from meristems using a combination of $0.2\text{--}4\text{ mg l}^{-1}$ benzyladenine (BA) plus 3 mg l^{-1} NAA; a combination of $0.2\text{--}4\text{ mg l}^{-1}$ kinetin plus $0.1\text{--}2\text{ mg l}^{-1}$ indoleacetic acid (IAA); or a combination of $0.2\text{--}4\text{ mg l}^{-1}$ kinetin plus $0.1\text{--}1\text{ mg l}^{-1}$ 2,4-D. Usually, the meristem explants died, but sometimes they grew up to 1mm and then died, irrespective of the combination used. Rost and Hinchee (1980), however, reported shoot growth from 0.5- to 1-mm apices using different combinations of auxins and cytokinins.

3.2 Shoot Proliferation

Proliferation or multiplication of shoots was performed by cutting shoots, (starting with those that developed from the initial explant) into nodal segments, each with two leaves and two axillary buds. The apical bud with the node below was also used.

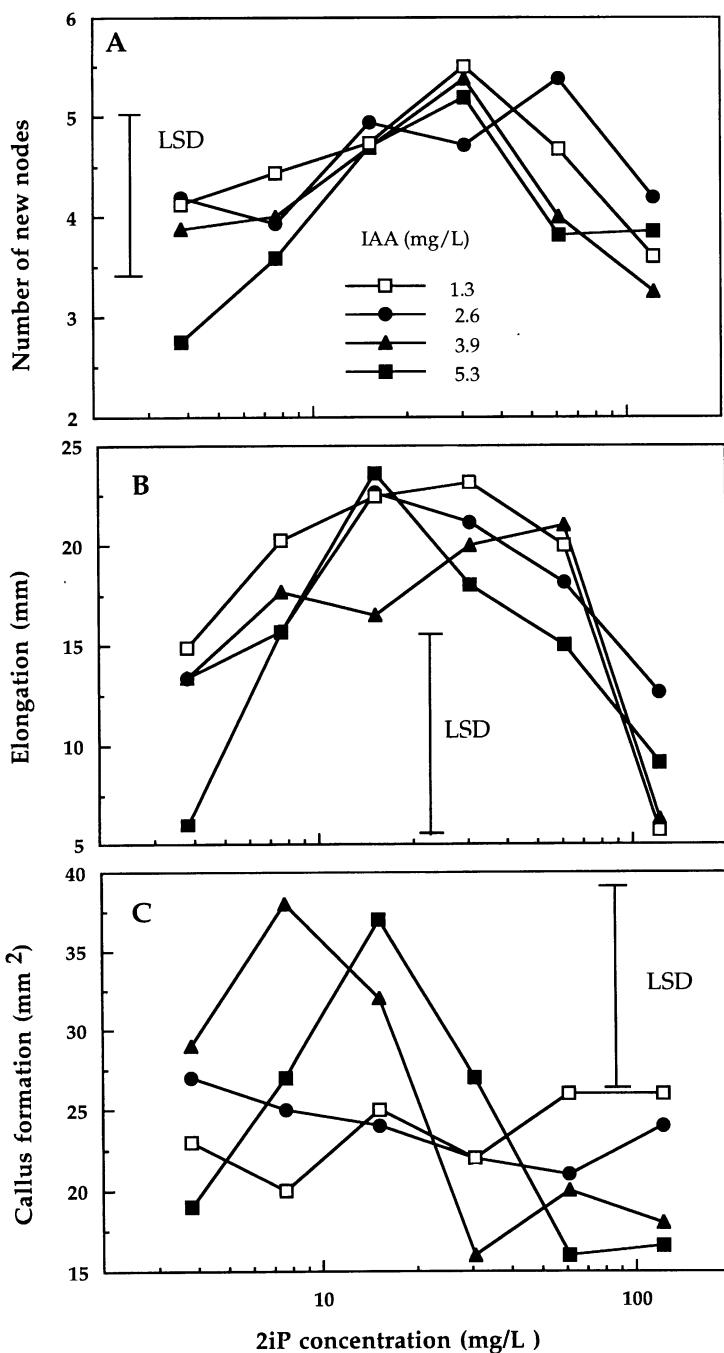


Fig. 4. **A** Effect of 2iP and IAA on node production; **B** elongation of shoots; **C** callus formation of nodal segments cultured for 8 weeks in the proliferation medium

Table 3. Growth parameters for apical and nodal segments. Values are means \pm SE for propagules of all treatments (see below)

Propagule		Total shoot length (mm)	No. of shoots	No. of nodes	Callus production (mm ²)
Type	No.				
Apical	191	23.1 \pm 0.9	1.83 \pm 0.11	5.2 \pm 0.1	25.7 \pm 1.0
Nodal	188	16.3 \pm 0.8 *	1.79 \pm 0.07	4.4 \pm 0.1	23.3 \pm 1.1
P		***	ns	***	ns

ns, Not significant.

*** $p < 0.0001$.**Fig. 5.** Typical shoot growth and callus of a nodal segment on the proliferation medium. The very small leaves are typical of clone 879-154. Shoot length is 45 mm. Note the two branches developing at the right leaf axil

In order to determine optimal conditions for shoot proliferation of both apical and nodal segments, hormonal combinations of 2iP (up to 122 mg l⁻¹) and IAA (up to 5.3 mg l⁻¹) were tried. The two types of propagules responded similarly to the various combinations of IAA and 2iP, but apical segments yielded longer shoots and more nodes than nodal ones (Table 3). The optimal 2iP concentration for the development of nodes and shoots (Fig. 4A) and for shoot elongation (Fig. 4B) in nodal and apical segments (data not shown) was 30.5 mg l⁻¹. IAA stimulated the production of callus at the base of the shoot (Fig. 4C). It was therefore necessary to use a low concentration of IAA so that the development of callus could be kept to a minimum (Fig. 5). The optimal combination for propagule production was 0.3 mg l⁻¹ IAA with 30 mg l⁻¹ 2iP for proliferating jojoba shoots.

The following treatments were also tried and occasionally adopted in an attempt to optimize the conditions of the proliferation stage media:

1. Adenine sulfate (80mg l^{-1}) significantly stimulated shoot growth and development (data shown in Fig. 4 include adenine sulfate).
2. Gibberellic acid (GA_3) had a positive, but not statistically significant, effect on shoot growth (i.e., elongation, number of branches, and number of nodes).
3. A twofold reduction in the salt concentrations in the proliferation medium (Table 2) resulted in a 50% decrease in the number of shoots (vs. the full medium); an increase in the concentration of salts did not enhance shoot or node production.
4. Omission of inositol and vitamins, with the exception of thiamine HCl, from the MS medium did not reduce shoot development.
5. Changing the ratio of KNO_3 to NH_3NO_3 in the media did not significantly affect shoot development. However, the use of NH_3NO_3 as the sole source of nitrogen was inferior, probably due to potassium deficiency.
6. An increase in light intensity from $25\text{ }\mu\text{E m}^{-2}\text{s}^{-1}$ (one fluorescent bulb) to $65\text{ }\mu\text{E m}^{-2}\text{s}^{-1}$ (three fluorescent bulbs) resulted in chlorosis (yellowing) of 10% of the leaves. At $115\text{ }\mu\text{E m}^{-2}\text{s}^{-1}$ (six fluorescent bulbs) the chlorosis rate increased to 14%, and an additional 19% of the leaves became orange.

Nodal or apical shoot segments were routinely multiplied in our laboratory, in tubes containing 5ml of proliferation medium, covered with Parafilm and placed under one fluorescent bulb at $25 \pm 1^\circ\text{C}$. With this treatment the two opposite buds usually grew and elongated, usually one bud elongating between two- and fivefold more than the other one. Shoot number per propagule (nodal segment) varied between 2 and 4.2, with an average of 2.6 (Table 4). Sometimes, additional shoots developed from an axillary bud in the middle of a new shoot. In other instances, depending upon the clone, shoots developed at the base of the nodal segment, creating a cluster of three to five shoots (Fig. 5). Both in field-grown jojoba plants and in in vitro-grown shoots axillary buds are found in the nodes between the leaf axil and a branch that had developed at that node (independent of flower bud formation). These buds are known as accessory buds (Gifford and Foster 1988). It is therefore likely that the multiple shoots formed in vitro were axillary and not adventitious shoots, suggesting that the system is relatively genetically stable. Throughout the long period of multiplying and preserving the clones (20 years), we have not noted any morphological changes in the typical characteristics of the clones, such as shoot length and width, internode length, or leaf

Table 4. Growth parameters of shoots of 18 jojoba clones grown on a proliferation medium for 8 weeks. Mean values for each clone were obtained from 40 propagules

	Length of shoot (mm)	No. of shoots	No. of internodes	Length of largest leaf (mm)
Mean	32.7	2.6	6.2	3.6
Min	19.6	2.0	3.8	1.6
Max	55.1	4.2	8.7	5.5

size and shape. Variability among jojoba shrubs originating from in vitro propagation was low (as discussed below).

The length of the shoots varied between the clones: some elongated to as much as 6 cm after 8 weeks and others to as little as 2 cm (Table 4). The number of internodes produced after 8 weeks, which can be roughly considered as the multiplication rate, varied between four and nine, six being the average of 18 clones for 8 weeks. Some clones, like clone 64, were characterized by big leaves, while others had small leaves.

In other studies different cytokinins were shown to be potent in induction of shoot production. Madani et al. (1979) were able to proliferate shoot tips with 10 mg l^{-1} dimethylaminopurine. Chaturvedi and Sharma (1989) reported that BA was far more effective than kinetin for induction of shoot proliferation in axillary buds. In addition, kinetin increased the production of callus at the base of the stem. Similar observations were reported by Lee (1988). Under the optimal treatment with BA and IAA, each at a concentration of 1 mg l^{-1} , 80% nodal segment proliferation was observed (Chaturvedi and Sharma 1989). In the latter treatment about five offshoots were produced, with a maximum of eight per explant. On further subculture, this number increased to about 15, when the basal stalk portion was repeatedly subcultured after the adventitious shoots that had developed were clipped off. Lee (1988) observed that shoot proliferation from basal tissues of explants was readily induced with $0.1\text{--}0.2\text{ mg l}^{-1}$ NAA and $1\text{--}2\text{ mg l}^{-1}$ BA in certain clones, while in others no shoot multiplication took place.

3.3 Shoot Elongation

Shoots, 3 mm in length, were excised with the apical meristem from the proliferating cultures and were subcultured on an elongation medium, which contained 0.5% charcoal but no plant growth regulators (Table 2). The cultures were illuminated with $65\text{ }\mu\text{Em}^{-2}\text{ s}^{-1}$. The rate of shoot elongation was about 3 mm/week.

Before we integrated this growing stage into the system, it was impossible to acclimatize jojoba plants ex vitro, while some success was obtained with the inclusion of this stage. It may therefore also be considered as an in vitro hardening stage. It should be noted that the leaves of jojoba growing in vitro were hyperhydrated (vitrified). Although they did not have a translucent, glassy appearance, they exhibited anatomical abnormalities and physiological disorders compared with leaves that had developed in the field (Fig. 6A). In the in vitro formed leaves the parenchymatic layer did not differentiate into spongy and palisade layers, as in the leaves from field-grown plants, and only very few air cavities were present (Fig. 6B). The typical tannin-accumulating cells were rare and no, or very little, deposit of cuticle waxes could be detected on the leaf surface (Fig. 7B). The vascular bundles were not well differentiated into xylem, phloem, supporting tissue and other elements. In addition, stomata were not sunken in tissue-culture leaves, as opposed to those of field-grown leaves (Dror 1981).

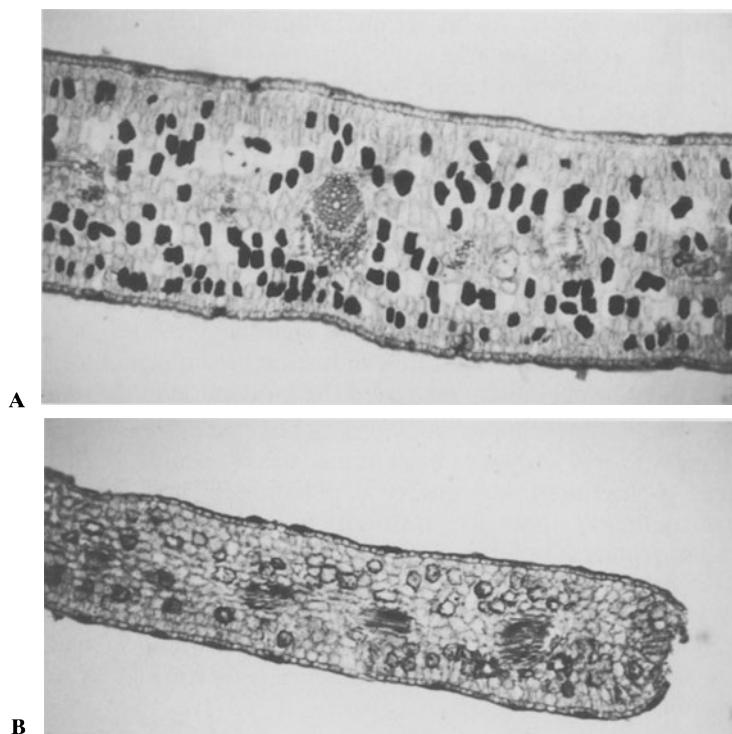


Fig. 6. Cross section through the leaf ($\times 52$). **A** A mature leaf from the field; **B** an in vitro leaf from the elongation stage

During the elongation stage some anatomical changes were observed. The outer cell wall of the epidermis and the stomata thickened. An outer ledge developed on the guard cells, and initial development of subsidiary (accessory) cells took place. Some wax was deposited on the leaf surface, and some air cavities appeared in the parenchymatic layer. The initial development of secondary cell walls in the xylem and phloem elements could already be detected. In addition, the chlorophyll content of jojoba leaves in the in vitro hardening stage increased by 33%, and their photosynthetic activity doubled in comparison to that of leaves in the proliferation stage (Dror 1981). In both stages the stomata were not effective in controlling water loss (Benzioni 1988).

In vitro hardening was induced to some extent by applying NaCl salinity in the proliferation stage. In some clones salinity resulted in the deposition of wax on the leaf surface (Table 5; Fig. 7C). The presence of wax on the leaves of NaCl-exposed nodal segments decreased the rate of water loss from detached shoots (Fig. 8).

3.4 Rooting

Under continuous exposure of shoots to NAA (up to 3.7 mg l^{-1}) and BA (up to 2.3 mg l^{-1}) rooting percentage was low, and large amounts of callus developed

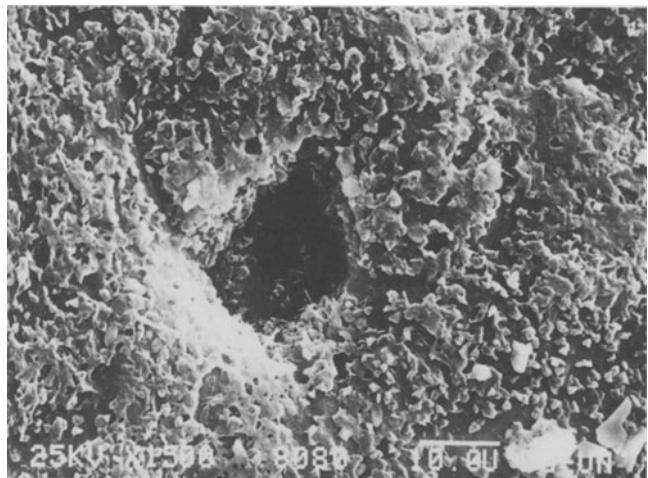


Fig. 7. Scanning electron micrograph of leaf surfaces. **A** A field-grown leaf; **B** an in vitro leaf from the proliferation stage; **C** an in vitro leaf from the proliferation stage in a medium supplemented with 0.6% NaCl

Table 5. Wax accumulation on the leaves of jojoba shoots grown on a proliferation medium supplemented with 0–1.2% NaCl. Scores, from no wax (–) to the maximum accumulation in this experiment (+++), were determined from scanning electron micrographs

NaCl (%)	Score for clone			
	879-154	78	64	Q63
0	–	–	–	–
0.4	–	+++	+++	–
0.8	–	++	++++	–
1.2	–	–	+++	++

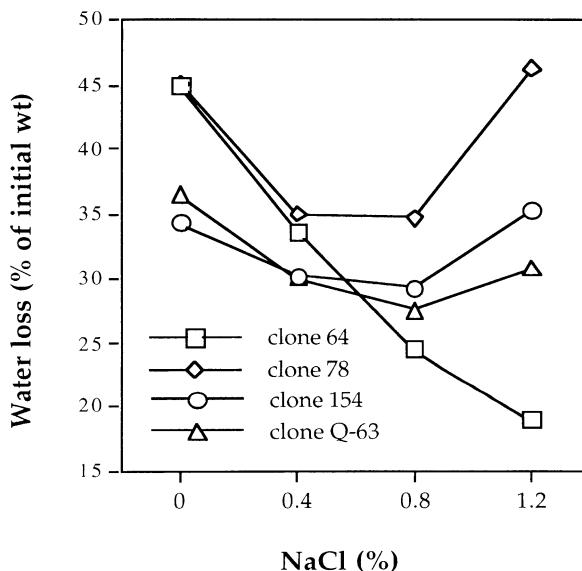


Fig. 8. Percentage of water loss after 60 min from detached jojoba shoots of four different clones grown under various NaCl concentrations. Water loss from a leaf taken from a field-grown plant was 3%

on the shoot base. Very often, browning of leaves was observed, followed by shoot death. A procedure of dipping shoots into a solution containing the rooting hormone (root induction), followed by subculturing the shoots in an auxin-free medium (root growth), was therefore adopted. Four different auxins, namely, IAA, NAA, 2,4-D, and IBA, with or without the addition of BA, were tested for root induction in a concentration range of 0–240 mg l⁻¹ and an exposure time of 12, 24 or 48 h. In general, at low or very high auxin concentrations no rooting occurred. At high auxin concentrations we observed damage (up to 60%) to buds and leaves (browning and death). The most potent

auxin for rooting was found to be IBA (120 mg l^{-1}); it induced 80% rooting, while NAA at the same concentration induced rooting of only 60% of the shoots. Callus production was observed at the base of 50–60% of the shoots exposed to IBA or NAA, and no elongation of the shoots occurred. Rooting percentage was much lower with 2,4-D or IAA. When BA was added to the root growth medium ($0.44\text{--}2.2\text{ mg l}^{-1}$), rooting was low; for example, in the presence of 1.35 mg l^{-1} BA only 38% of the shoots produced roots, and in all the shoots a large amount of callus developed at the shoot base. Thus, BA was also omitted from the root growth medium and was added only to the root induction medium (1.35 mg l^{-1}). A twofold decrease in the concentration of MS salts in the root growth medium increased rooting percentage. A 50% increase in the concentration of salts completely prevented rooting. Addition of charcoal (0.5% w/v) to the rooting medium had a significant positive effect on shoot growth. It also reduced callus growth, and although in 50% of the cultures some callus was still observed, the callus was significantly smaller in size. Sucrose concentrations up to 2% also improved rooting and inhibited callus growth (Fig. 9).

With the optimal protocol for rooting (Table 2), rooting percentage ranged between 20 and 95%, depending on the clone and the particular trial, and was usually 50–60%. We have recently begun using to Sorbarod system for rooting shoot segments, and it seems to be superior to the agar system (Fig. 10). There are indications that there is no need for charcoal on the cellulose plugs.

Chaturvedi and Sharma (1989) reported that a combination of two auxins, 1 mg l^{-1} NAA plus 7 mg l^{-1} IBA, was more effective for rooting than the appli-



Fig. 9. Typical root development in the root growth medium. Shoot length is 30 mm. The very big leaves are typical of clone 64



Fig. 10. Root development in a Sorbarod cellulose plug. The plantlet is ready for acclimatization

cation of either one alone. Addition of 2mg l^{-1} chlorogenic acid or 1mg l^{-1} caffeic acid further increased rooting percentage from 40 up to 70%, or sometimes even to 90%. In all the above-mentioned treatments three to four roots developed in each shoot along with a significant amount of callus. According to Chaturvedi and Sharma (1989), this type of root system did not facilitate good acclimatization upon transplanting into soil. In order to obtain a single thick root developing directly from the bottom of shoot with no intervening callus they halved the mineral concentration of KNO_3 , MgSO_4 , and CaCl_2 to 500, 200, and 100mg l^{-1} , respectively. An additional improvement in the rooting percentage of this type of root was achieved by increasing the sucrose concentration from 2 to 7% and by transferring the shoots to an auxin-free medium after 15 days.

3.5 Acclimatization

Acclimatization of rooted jojoba plantlets directly into the greenhouse was first attempted. Rooted plantlets were dipped in 0.5% Benlate and 5% Folicote and transplanted into Styrofoam speedling trays or paper sleeves (both $5 \times 5\text{cm}$), containing a 1:1 sterile mixture of peat moss and perlite, or 1:1:1 peat moss, vermiculite, and perlite. Plantlets were covered with glass jars and placed on heated tables covered with netting in a temperature-controlled greenhouse, equipped with mini-sprinkle irrigation. The jars were kept over the plantlets for at least 1 month, and then the surviving plants were transplanted into polyethylene bags containing a mixture of 1:1:1:1 sand,

sandy loam soil, perlite, and peat moss. The temperature fluctuated in the greenhouse between 20 and 33°C (and sometimes rose to 39–41°C), with humidity between 45 and 90%. The survival rate of plantlets ranged between 0 and 45%, but was usually quite low. For example, out of 6352 plantlets that were transplanted during 1980, only 461 grew successfully (7%). Survival rate could be manipulated to some degree by certain treatments, such as the use of antitranspirants, addition of mycorrhiza fungus, and new soil substrate combinations, but unfortunately it was influenced mostly by environmental conditions and the clone itself. In addition, the jojoba plants in the greenhouse were also affected by fungal diseases, insects, and nematodes, which were only partially controlled by periodic spraying.

The use of an environmental chamber (temperature 25°C, and humidity at 90% for 2 weeks and 22°C and 80% for an additional 3 weeks) before transfer to the greenhouse proved beneficial. Rooted plantlets were kept in the in vitro rooting vessel for 3 days in the growth chamber, before the Parafilm cover was removed, and only 2 days later were the plantlets transplanted into the soil mixture and covered with jars. The jar protection was gradually reduced by removing the jars for about 1 h on the first day, 2 h on the second, and so on. Plants were usually transferred to the greenhouse 5 weeks after transplanting. By following these steps we obtained 50–90% survival, with an average of about 80%. In a parallel experiment with the same plant material, but without the growing chamber stage, survival was only 30%. The improved control of temperature of humidity or both was probably the reason for better survival in the environmental chamber.

A procedure developed by Chaturvedi and Sharma (1989) was based on a gradual reduction of humidity in five steps over 2 weeks, as follows: (1) plantlets were transplanted into a soil mixture and covered with a vertical glass cylinder (12 cm long) closed with a lid; (2) after 1 week the short cylinder was replaced with a long one (21 cm long); (3) 4 days later the lid was taken off; (4) after 2 more days the long cylinder was replaced with a the short one; (5) and finally 2 days later the glass cylinder was removed. In this system water logging of the soil was prevented, allowing good aeration, while humidity around the shoot which was initially high, was gradually decreased. Details on the environmental parameters such as light, temperature, and humidity were not reported by Chaturvedi and Sharma (1989). Similarly, Dunstone (1992, pers. comm.) found it necessary to decrease humidity gradually in order to acclimatize rooted or unrooted plantlets. He used the following procedure: shoots were dipped into IBA and rooted in plastic boxes covered with a plastic film wrap. Four to six holes were pierced every few days for several weeks until the film could be removed completely.

The three acclimatization systems mentioned above are the only ones giving a reasonable percentage (about 80%) of survival, but they are complicated and seem unpractical for mass propagation. A better controlled greenhouse that keeps temperatures low enough and permits high humidity around the canopy but relatively low humidity in the soil substrate is crucial for mass acclimatization of jojoba plantlets. We are currently studying treatments like gradual reduction of humidity in the culture tubes and the use of

polyethyleneglycol as an osmoticum in order to enhance in vitro hardening. It should be noted that roots of jojoba are fragile and sometimes break upon transfer to soil substrate, a factor that contributes greatly to the difficulties of acclimatizing jojoba plants. The use of the Sorbarod system is expected to be useful in this aspect of micropropagation.

4 Field Performance of Tissue-Culture Produced Plantlets

About 20 000 micropropagated rooted plants were produced in our laboratory during 1978–1984 and of these about 2000 survived and were transplanted into three locations in Israel: the grounds of the Institutes, Kibbutz Givat-Brenner, and Kibbutz Hazerim (Fig. 11).

The plot that was best maintained and monitored was that at the Institutes. The yield performance of plants of two clones originating from shoot cultures is given in Table 6. Yields of 2.7 and 2.8 kg/plant for the two clones at 5 years of age is similar to the yield obtained from vegetative cuttings at the same age (Benzioni 1995). There was variability in some of the phenological characteristics between the plants (Table 7). The degree of variability between plants, however, was usually smaller than the variability within the plants themselves, suggesting that the plants are true to type.



Fig. 11. Four-year-old jojoba plants of superior female clones grown at Kibbutz Hazerim. Seeded plants (second left from center) were incorporated every 15 rows as a source for pollen

Table 6. Yield data for plants of two clones raised by tissue culture. Plants were planted in October 1980 on the grounds of our Institutes, Beer-Sheva, Israel. Both clones were obtained from mother plants selected in the Gilat plantation on the basis of their shape and yield. The mature mother plants had 10-year average yields of 3765, and 2625 g/plant for clones Q-106 and 879-154, respectively. Each value is an average of 24 (879-154) or 28 (Q-106) plants

	Yield (kg/plant) for clone Q-106				Yield (kg/plant) for clone 879-154			
	1983	1984	1985	1986	1983	1984	1985	1986
Average	0.22	0.44	1.26	2.73	0.53	1.16	1.88	2.82
Range	0.13–0.38	0.13–0.69	0.45–2.30	1.80–5.30	0.07–0.80	0.50–1.79	1.00–2.80	1.80–4.20

Table 7. Statistical data on some phenological characteristics of two clones raised by tissue culture. Mean values were obtained for 17–20 measurements for all 24 plants of clone 879-154 or 28 plants of clone Q-106 plants. Variance between and within plants (residual) was calculated from one-way ANOVA and presented as percentage of the total variance. (For additional data see Table 6)

Phenological characteristic	Clone		P
	Q-106	879-154	
Seed length/width			
Mean	1.76	1.81	0.0001
Range	1.67–1.92	1.66–1.92	
s^2 Within plants (%)	91	85	
s^2 Between plants (%)	9	15	
Peduncle length (mm)			
Mean	8.5	4.9	0.0001
Range	6.3–12.7	2.0–7.8	
s^2 Within plants (%)	91	64	
s^2 Between plants (%)	9	36	
Internode length (mm)			
Mean	32.0	26.7	0.0001
Range	26.8–35.3	19.6–34.8	
s^2 Within plants (%)	99.5	76	
s^2 Between plants (%)	0.5	24	

5 Selection for Salt Tolerance

Screening shoot cultures for NaCl sensitivity revealed considerable variability in the response of some of the clones propagated in our laboratory (Fig. 12): in some clones shoot elongation was inhibited by 70–80% by the presence of 1% NaCl in the proliferation medium, while other clones were not affected. A study conducted ex vitro with the same clones as those used in the above-mentioned in vitro experiment showed that the response of shoots to salinity in vitro was similar to that of young potted plants in a nethouse (Benzioni et al. 1992; Mills and Benzioni 1992).

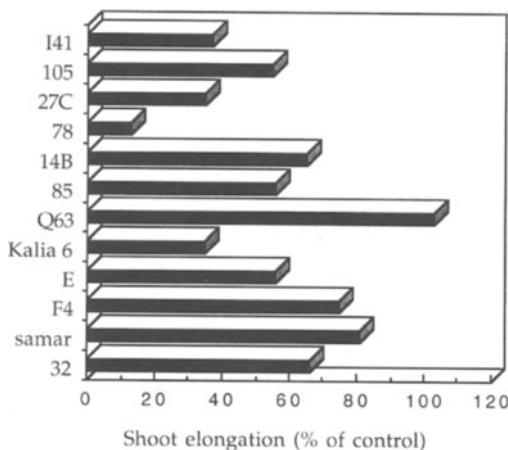


Fig. 12. Effect of 1% NaCl on shoot elongation in the proliferation stage (as percent of the control treatment) of different jojoba clones. Values are means of 40 nodal segments per clone

6 Conclusions and Prospects

Shoot culture initiation of jojoba clones was successful when small explants consisting of an apical or axillary bud with some underlying shoot tissue were used. With such explants fungal contamination was not significant, unlike the situation with bigger explants. It was possible to establish all clones in different seasons with as little as eight explants, provided that the explants were taken from active growing stems. The observation that axillary, and not adventitious, buds developed into shoots during the proliferation stage and the low variability between jojoba plants originating from tissue culture suggests that the propagation system is relatively genetically stable.

A shoot elongation stage was added to facilitate in vitro hardening. The best regime for induction of rooting was found to involve dipping shoot segments in IBA and then culturing them on an hormone-free medium supplemented with charcoal.

Acclimatization of jojoba is the most difficult stage, presumably as a result of leaf hyperhydration. The lack of epicuticular wax in combination with incomplete development of the vascular system plus malfunction of the stomata lead to rapid water loss and consequent plant death. Nevertheless, relatively high success was obtained by acclimatizing plantlets in a controlled environmental chamber before transferring them into the greenhouse, or by gradually decreasing humidity around the plant canopy. In the past, unsatisfactory survival rates during acclimatization was the main reason for the failure of commercial mass micropropagation of jojoba in Israel. If acclimatization can be simplified, production costs can be lowered, and survival rates can be increased then commercial production may be resumed. Simplifying

the acclimatization stage via improving in vitro hardening is currently being undertaken in our laboratory.

It should be noted that in Israel some of the three- and four-year-old plantations originating from cuttings have been infected by *Fusarium oxysporum* (sometimes up to 30%). It is suspected that this disease was transmitted by the cuttings which originated from infected mother plants. Microppropagation may enable the establishment of disease-free plantations and prevent such problems.

Shoot culture may be used for screening of clones for resistance to particular factors, such as salinity or diseases. We already have good indications that response to salinity of shoots grown in vitro is similar to that of young plants of the same clones grown ex vitro.

7 Protocol for Microppropagation

1. Take explants from selected mother plants in a state of vigorous growth. Rinse the tips of the branches (about 10cm long) in tap water containing a liquid detergent. Divide each cutting into single-node (or apical) segments. Subject the segments to surface sterilization for 10 min in 1% NaOCl plus one drop of Tween 20 per 100ml. Wash three times with sterile deionized water.
2. Excise the vegetative axillary or apical buds together with a small amount of shoot tissue. Culture the buds directly on the proliferation medium at 25°C under a light intensity of 25 $\mu\text{E m}^{-2}\text{s}^{-1}$ and a photoperiod of 16h (light)/8h (dark). Subculture nodal and apical segments on the proliferation medium every 4–6 weeks.
3. Select and excise 3-cm-long shoots with their apices from the proliferation cultures, and culture them on an elongation medium at 25°C (16-h/8-h photoperiod, 65 $\mu\text{E m}^{-2}\text{s}^{-1}$).
4. After 6–8 weeks, remove the callus, if any, from the base of the shoot, and renew the cut at the base. Transfer shoots to small tubes (e.g., 16 × 100 mm) containing 0.5 ml of the liquid root induction medium at 25°C (16-h/8-h photoperiod, 25 $\mu\text{E m}^{-2}\text{s}^{-1}$).
5. After 24 h transfer shoots to the root growth medium at 25°C (16-h/8-h photoperiod, 65 $\mu\text{E m}^{-2}\text{s}^{-1}$). Roots should start to appear after 2 weeks.
6. At the site of acclimatization, remove lid or cover of the vessel of rooted plantlets for 2 days. Then clean the roots gently from agar; treat the plantlets with 0.5% Benlate and 5% Folicote; and pot in a mixture of peat moss:vermiculite:perlite (1:1:1). Cover plantlets with glass beakers leaving the periphery area of the soil uncovered. Place plantlets in a controlled environment, preferably 25°C and 90–100% humidity for 18 days. The soil mixture should be moist, but water logging must be prevented. Remove the jars gradually, for about 1 h on the first day, 2 h the next day, and so on. On day 21 remove the jars completely.
7. The different periods described above and the actual times at which to transfer the plants to lower humidity in the greenhouse or nethouse have to be fixed by ongoing experimentation.

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