In vitro grafting of woody species

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Abstract

In vitro grafting is a relatively recent vegetative propagation technique which consists of grafting miniaturized scions in axenic culture conditions. It combines thus the advantages of grafting and of shoot tip culture, while overcoming certain limitations of these two methods. Several in vitro grafting procedures can be considered, differing one from another by the type of grafting technique used, and also by the characteristics of the in vitro rootstock and scions involved. Initially developed for eradicating endogenous pathogens from fruit tree species and cultivars, in vitro grafting has been rapidly extended to various woody species in different domains of plant physiology and improvement. These encompass grafting incompatibility and also physiological rejuvenation as a prerequisite for mass clonally propagating mature selected genotypes of most woody species. In conclusion, in vitro grafting is an original and skilful technique which should deserve more consideration for overcoming the limitations of more common other vegetative propagation methods, and also for studying more in depth the relationships between genetically different tissues and cells.

Key words: graft, micrografting, rejuvenation, scion, shoot tip, tissue culture, vegetative propagation.

INTRODUCTION

In vitro grafting is a relatively new vegetative propagation technique developed in the early 1970's primarily for eliminating viruses different fruit tree species, starting with *Citrus spp* (Murashige et al 1972, Navarro et al 1975), before application to others (Colin and Verhoyen 1976, Huang and Millikan 1980, Jonard et al 1983), including ornamental ones (Crézé 1984). It has been subsequently utilized with the prospects of physiologically rejuvenating mature selected genotypes, mostly of trees species, with a view to stimulate their capacity for true-to-type cloning by rooted cuttings (Tranvan and David 1985, Monteuuis 1986, Dumas et al 1989). In vitro grafting permits to graft miniaturized scions, usually shoot tips ranging from 100 µm to 1 or 2 cm in total length, too small to be successfully grafted in in vivo conditions where fungus damages, desiccation or browning of the cut areas remain unavoidable constraints. For certain species, these shoot tips can be restricted to the shoot apical meristem, or SAM *per se*. This latter consists of the apical dome plus one or two leaf initia or primordia, and its overall size is liable to vary according to the species and also to the physiological stage and plastochron (Romberger 1963, Mankessi *et al* 2010). In vitro grafting combines the advantages of grafting (Hartmann et al. 1997), and the benefits of SAM tissue culture (Bonga and Von Aderkas 1992, George 1993), while overcoming basic limitations of these two vegetative propagation techniques.

Grafting allows:

- the cloning of individuals considered remarkable with regard to particular quantitative or qualitative criteria such as growth, form, wood, fruit and flower characteristics, ornamental features, but that cannot be propagated by rooted cuttings due to an insufficient capacity for adventitious rooting;

- to reproduce true-to-type special forms, growth habits or developmental patterns such as bushy, compact or pendulum cultivars particularly praised for ornamental of landscaping utilizations:
- to reduce growth vigor for increasing plantation density and yield in fruit tree orchards for instance;
- to replace the initial root system of the selected genotypes to be clonally propagated by a more appropriate one coming from the rootstock;
- to modulate physiological ageing in two main ways: (i) hastening flowering and fruiting onset for ornamental as well as production purposes in seed and fruit orchards, (ii) rejuvenating mature selected genotypes with a view to their mass propagation by rooted cuttings (Franclet 1983, Hackett 1985).

In addition to these advantages, in vitro grafting can be used for rescuing somatic or nucellar embryos (Huang et al 1988, Raharjo and Litz 2005), as well as for studying the histological and physiological aspects of grafting. It can be also helpful for overcoming certain incompatibility problems that may arise from traditional grafting, as developed thereafter.

SAM in vitro culture is useful for initiating contamination-free tissue cultures while eradicating endogenous contaminations especially from virus origins (George 1993). It has been also reported to induce physiological rejuvenation of mature selected genotypes of different trees species, which remains a prerequisite for their mass clonal propagation by rooted cuttings (Hackett 1985, Monteuuis 1989, Bonga and Von Aderkas 1992). For these purposes, the smaller the shoot tip, the more effective the elimination of pathogens and also the physiological rejuvenation, but on the other hand the less chances of culture success (Bonga and Von Aderkas 1992, Bonga et al 2010). However, in vitro culture of SAMs remains unsuccessful for a large number of woody species (George 1993, Bonga et al 2010). This seems more likely due to the inappropriateness of the tissue culture synthetic media used according to the species, SAMs

becoming increasingly more medium-sensitive as the donor tree from which they derive ages (Nozeran 1984, Monteuuis 1987a). In vitro grafting constitutes an elegant and helpful alternative to these limitations, replacing the unsuitable synthetic culture media by a more natural one, the in vitro raised rootstock. Moreover, the hypothetical influence of beneficial rejuvenating substances produced by the juvenile seedlings used as rootstocks on the micrografted SAMs cannot be ruled out (Bon and Monteuuis 1987, Bon 1988, Huang *et al* 1992a).

The successfully in vitro grafted plants can be acclimatized to outdoor conditions for further utilization, or kept in vitro for producing healthy and responsive shoots that can be used for further serial grafting or micropropagation cycles on synthetic tissue culture media.

The various in vitro grafting techniques as well as their main applications are being reviewed in this paper.

METHODOLOGICAL ASPECTS

Rootstock production

Usually in vitro germinated seedlings of the same species as the grafted scions are used as rootstocks, giving rise to homografts; more seldom, different species can be utilized to produce then heterografts (Navarro et al 1975, Jonard et al 1983). For various *Picea spp* and cultivars, Ponsonby and Mantell (1993) did not observe any significant difference in grafting success rates between in vitro homografts and heterografts. Regardless of rootstock identity, the culture medium must be adapted to accurate, skilful and harmless manipulations under binocular microscope in axenic conditions. Several systems of rootstock production have been tested (Alskief and Villemur 1978, Navarro et al 1975, Monteuuis 1987), including liquid media (Gebhardt and Goldba 1988), with possible use of sterilized filter paper bridges for better maintenance (Murashige et al 1972, Navarro 1988, Huang et al 1988, Sanjaya et al 2006).

Perlite (Crézé 1984, Monteuuis 1987) and vermiculite (Jonard et al 1983, Tranvan and David 1985, Monteuuis 1987) could be preferable to liquid media as less suffocating than totally liquid media for stock root systems. Polypropylene fiber (Deogratias et al 1986, Monteuuis 1986, 1987, Ponsoby and Mantell 1993, Danthu et al 2002a and b, 2004), then cellulosic (Dumas et al 1989, Monteuuis 1994, 1995a, 1996) plugs, or peat-pellets (Ewald and Kretzschmar 1996) were also used. The main advantage of these plug supports compared to usual gelled culture media lies in the possibility to pull out from the culture tube, for easier grafting manipulations, the in vitro rootstock together with its plug support without damaging the root system.

Rootstock developmental stage and vigor have been observed to promote micrograft success in various species such as *Citrus spp* (Navarro et al 1975, *Prunus persica* (Mosella et al 1980), *Sequoiadendron giganteum* (Monteuuis 1987), *Picea spp* (Ponsonby and Mantell 1993) and *Santalum album* (Sanjaya et al 2006). Embryos dissected from imbibed seeds then placed horizontally on gelled culture media appeared quite promising for micrografting SAMs from mature white pines (Goldfarb et al 1992, Monteuuis unpublished results). For some fruit trees species, the cotyledons are removed from the young rootstock seedlings (Jonard 1986). In *Sequoia sempervirens*, Huang et al (1992a) micrografted shoot tips onto in vitro seedling-derived microshoots just after their root induction but prior to root emergence.

Preconditioning the in vitro seedlings used as rootstock by maintaining them in total darkness for a few days prior to grafting was recommended by Navarro et al (1975) for citrus. Addition of various growth regulators in the culture medium was reported to increase also grafting success of different fruit tree species (Jonard et al 1987).

Scion characteristics

Type

Usually shoot tips are preferably used as scions, but in pines, newly initiated brachyblasts still protected by bud scales (Monteuuis, unpublished results), or fully developed but trimmed to graft only the 1cm long basal part including the interfascicular meristem (Cortizo et al 2004), were also successfully utilized for in vitro grafting. Scion size is liable to vary from 0.1 mm to 2 to 3 cm in length according to the species, the manipulators and also to the technique used. As examples, in *Sequoiadendron giganteum*, 35% of micrografting success was obtained for resting SAMs of 0.4mm as overall size collected from 100 yr-old individuals (Monteuuis 1986). In *Picea abies* and *Pseudotsuga menziesii*, micrografting SAMs of 0.2 to 0.45 mm in width for 0.1-0.25mm (or 0.5mm when removed with the basal wedge suitable for side-grafting) in height resulted in average success rates of 50% (Monteuuis 1994, 1995a). In vitro grafting 140 yr-old *Larix decidua* trees, Ewald and Kretzschmar (1996) obtained 69% of success when using 0.3 to 0.5 mm wide scions consisting of the SAM plus the first ring of needle primordia underneath. In *Sequoia sempervirens*, Huang et al (1992a) reported that 1.5 cm long shoot tips scions gave the best micrografting success rates. In vitro grafting 1 to 2 cm long shoot tips in cashew resulted in 60 to 80 % success rates (Mneney and Mantell 2001), while in *Acacia senegal*, Khalafalla and Daffalla (2008) obtained 70 to 90% of micrografting success for shoot tips ranging between 1.5 and 3 cm in length. For micrografting *Prunus avium* and 20 yr-old *Garcinia indica* trees, Deogratias et al (1986) and Chabukswar and Deodhar (2006) recommended *in vitro* microshoots tips of 0.4 to 1mm and 0.5 to 1cm in length, respectively.

The rationale of reducing scion size to the SAM for rejuvenation and phytosanitation purposes is argued thereinafter. However, it seems that practically the minimal size of the scion to be grafted with high enough success rate averaged 0.1-0.2 mm depending on the species, the manual dexterity the manipulator – the scions need to be excised and micrografted quickly and carefully in aseptic conditions -, and also on the micrografting technique used. For instance

146 excising the scions with a wedge of underlying tissues can facilitate its insertion into the
147 rootstock tissues. Scions of 0.1 to 0.2mm in size can correspond to the apical dome for certain
148 species, or to shoot apices in others like *Acacia mangium* or *Eucalyptus spp* characterized by
149 smaller SAMs (Monteuuis 1996, Mankessi et al 2010). Attempts of reducing scion size of 0.4 to
150 0.8mm to less than 0.2mm resulted in a dramatic decrease of micrografting success rate in
151 *Acacia mangium* (Monteuuis 1996). In *Sequoiadendron, giganteum*, grafting SAMs of 0.2 to
152 0.3mm as overall size resulted in 23% of survival rate versus 61% when 0.4 to 0.5mm shoot
153 apices were used (Monteuuis 1987). In *Citrus spp*, Navarro et al (1975) also noticed that
154 grafting success decreased progressively as scions size diminished, from 47% for 0.4-0.7 mm
155 long scions to 1.8 % when 0.05 to 0.1 mm long scions were used. Likewise, in vitro grafting of
156 apple trees, gave 18% and 3% of success for scions of 0.5-0.8 mm and less than 0.1 mm in
157 length, respectively (Huang and Millikan 1980), whereas for the same species, Alskief and
158 Villemur (1978) obtained average success rates of 26 % with 2 to 3 cm long scions. It can be
159 logically assumed that the smaller the scion, the greater the stress of the excision cuts from the
160 donor plant and the less resource available to survive until the connection with the rootstock
161 tissue becomes efficient. Besides, micrografting tiny scions in axenic conditions require higher
162 dexterity and longer time than for bigger ones. Manipulator dexterity could explain that in *Pinus*
163 *pinater*, Tranvan and David (1985) obtained 43 to 50% versus 0% of success for shoot tip
164 scions of 5-7 mm and 2-3 mm in length, respectively, whereas for the same species Dumas et
165 al (1989) recorded 60 to 80 % of success rates when micrografting SAMs of 0.4 mm in size from
166 older trees. In *Camelia japonica* cultivars, shortening scions size from 8 mm to 3-5mm in length
167 resulted in a diminution of average grafting success rates from 67% to 8% (Crézé 1984). In
168 *Prunus spp*, grafting scions of less than 0.4mm in length failed totally, while the best grafting
169 scores were obtained for 0.4-0.6mm long scions (Colin and Verhoyen 1976). In *Santalum*
170 *album*, reducing shoot tip length from 1-2 cm to 0.4 - 0.5 cm induced a decrease of cleft-micrografting

171 success, from 24% to 4% (Rai 2003), and from 60% to 10% (Sanjaya et al 2006), respectively
172 depending on the experimental conditions. For different tree species, Jonard et al (1983)
173 recommended to cultivate in vitro during two weeks the 0.2-0.4 mm long shoot apices (< 1 mm)
174 excised from the donor tree to be in vitro grafted, in order that the scions could reach 1 cm in
175 length at the time of grafting for easier manipulations and higher success rates. Applying this
176 pre-treatment to 0.4mm long *Citrus spp* shoot apices, Jonard et al (1987) obtained ultimately
177 100% of grafting success after successive improvements of the grafting technique. In *Prunus*
178 *dulcis*, Yildirim et al (2010) did not notice any significant difference of micrografting success
179 between scions ranging from 4, 8 and 15 mm in length, but observed that the smaller the scion,
180 the shorter the shoot produced 28 days after grafting.
181
182 **Genotypic origin**
183 In vitro grafting success has been observed to vary between and within species. The
184 micrografting procedures successfully developed for various coniferous species (Monteuuis
185 1986, Dumas et al 1989, Monteuuis 1994 and 1995a) have been observed to work also for
186 *Acacia mangium*, but not for *Tectona grandis* and *Eucalyptus spp* despite similar experimental
187 conditions and with the same operator (Monteuuis unpublished results). Ponsonby and Mantell
188 (1993) noticed that *Picea pungens 'Koster'* had a higher ability for micrografting (43%) than
189 *Picea abies* (7%), for which a much greater proportion of wounded tissues became brown
190 quickly.
191 At the within species level, significant differences of micrograft survival then of scion
192 elongation rates could be noticed between genotypes of the same age in *Pinus pinea* (Cortizo et
193 al 2004) and in *Pinus pinaster* (Dumas et al 1989) using respectively needle fascicles and SAMs
194 as scions. Shoot tip micrografting was reported to vary noticeably also in *Citrus spp* between the
195 various combinations of scions and rootstock cultivars tested (Navarro et al 1975, Edriss and

196 Burger 1984, Kapari-Isaia et al 2002). In grapevine (*Vitis vinifera*) amazingly, such variations of
197 grafting success according to the genetic identity of the various scions and rootstock cultivars
198 grafted were not significant in vitro, but more obvious for conventional in vivo grafting
199 (Pathirana and McKenzie 2005).
200
201 **Physiological condition**
202 Substantial within clone variability of in vitro grafting success and further scion
203 elongation according to the date of collection from the outdoor donor plant was reported for
204 micrografted SAMs of several species including *Sequoiadendron giganteum* (Monteuuis 1986),
205 *Pinus pinaster* (Dumas et al 1989, Monteuuis an Dumas 1992) and *Picea abies* (Monteuuis
206 1994). In *Malus domestica*, micrografting 0.1-0.2mm shoot apices resulted in 10% of success
207 for scions collected from outdoors during the November-March period, increased to 70% in May,
208 to decrease of about 10 % per month from June through October (Huang and Millikan 1980).
209 Using brachyblasts as scions, Cortizo et al (2004) in *Pinus pinea*, and Fraga et al (2002) in
210 *Pinus radiata* noticed also seasonal variations of in vitro grating success, the best scores
211 corresponding for this latter species to winter collection, during the resting period. In
212 *Sequoiadendron giganteum*, SAM micrografting was more successful also for meristems
213 excised from resting shoots (52%) than from elongating ones (26%) (Monteuuis 1987), and
214 likewise for *Prunus avium* shoot apices (Deogratis et al 1986). By contrast, it seemed that for
215 rhythmic growth species like *Picea spp*, better grafting results could be obtained for growing
216 shoot tips than for resting ones (Ponsonby and Mantell 1993).
217 In *Prunus persica*, the first micrografting attempts resulted in average success rates of
218 14 % (Alskief 1977). Subsequent studies established that in vitro grafting success varied
219 markedly according to the period of scion removal from the outdoor donor plant, the best scores
220 corresponding to June collections. Close relationships with the peroxydase content of the

grafted material suggested a very likely influence of auxin metabolism co-factors on micrografting response (Poessel et al 1980, Jonard 1986).

For certain species like *Malus domestica* (Huang and Millikan 1980), *Prunus avium* (Deogratias et al, 1986), *Ziziphus mauritiana* (Danthu et al 2004), *Acacia tortilis* (Detrez 1994) and *Garcinia indica* (Chabukswar and Deodhar 2006), *Protea cynaroides* (Wu et al 2007), the use of *in vitro* microshoot tips as scions seemed preferable to outdoor materials.

It can be logically assumed that these time-related fluctuations of micrografting responsiveness are induced by variations of scion physiological status at the time of removal from the donor plant. For various fruit tree species, the pretreatments recommended by Jonard et al (1983) included also exogenous application of zeatin, aiming at improving scion physiological conditioning and hence grafting success. For *Citrus spp* more specifically, Jonard et al (1987) advised to maintain the scions on MS basal culture medium supplemented with GA3, BA and 2, 4 D in complete darkness during 5 days, then under a 16/8 photoperiod regime for 2 days prior to grafting. Pre-treating the 0.15-0.25 mm long scions with 2,4-D or kinetin, or with the combinations of these two growth regulators was reported to enhance noticeably grafting success rates for different citrus cultivars used as scions or rootstocks (Edriss and Burger 1984, Burger 1985).

Age

The influence of the age of the donor plant from which the scions had been collected on in vitro grafting success has been considered for several species. In *Pinus pinaster,* Tranvan and David (1985) using 5 to 7 mm long shoot apex scions obtained similar micrografting success rates for 2-3 month-old seedlings (43%) as for 11 yr-old trees (45%). Micrografting SAMs from 11 yr-old and 80-100 yr-old trees of the same species gave overall higher and comparable survival rates (65 to 80%) for the two age classes, but the scions collected from the

246 younger trees resumed growth quicker and in greater proportions than those derived from the
247 older individuals (Dumas et al. 1989). In *Pinus radiata*, micrografted brachyblasts from young
248 seedlings elongated in significantly higher proportions than those removed from mature trees,
249 but the percentage of successfully established grafts was not influenced by the age of the donor
250 plant (Fraga et al 2002). Interestingly, immature brachyblasts (3 to 5 week-old) of the same
251 species showed a higher capacity for micrografting than more developed ones (12 month-old)
252 (Valledor et al. 2010). In *Picea abies*, Ewald et al (1991) observed similar side-micrografting
253 success and scion elongation scores for scions collected from 11, 19 and 55 yr-old trees.
254 Grafting in vitro 5 to 8 mm long *Acacia tortilis* shoot tips gave higher rates of scion survival and
255 subsequent growth for 2 day-old seedlings than for 11 yr-old donor trees (Detrez 1994). In
256 *Acacia mangium*, similar micrografting success rates were reported for 0.3 to 0.4mm long shoot
257 apices from young seedlings (52%) and mature donor trees (46%), whereas the same sources
258 of plant materials grafted in in vivo conditions using bigger scions resulted in 49% and 0% of
259 grafting success, respectively (Monteuuis 1995b). Here again, the juvenile micrografts seemed
260 to elongate faster than those issued from the mature plants. Using 5-10mm long pistachio
261 (*Pistacia vera*) shoot tips as scions, Onay et al (2003) noticed a marked influence of donor plant
262 age on grafting success and on elongation of the successfully established micrografts, as
263 illustrated by the following records 2 months after grafting (donor plant age - grafting success
264 rate - mean shoot length): 1yr-old -14/20=70% - 7.8cm; 5 yr-old - 9/20=45% - 6.7cm; 10 yr-old -
265 5/20=25% - 5.1cm; 30 yr-old - 4/20=20% - 4cm.
266
267 ***Grafting technique***
268 The in vitro grafting manipulations must be carried out in axenic conditions, usually
269 under a laminar flow hood and a binocular microscope equipped with a cold light source.
270 Different in vitro grafting techniques deriving in fact from the miniaturization of more classical

grafting procedures (Hartman et al. 1997) have been developed (Fig 1, 2 and 3), with varying success rates depending on the species.

In vitro side-grafting (Monteuuis 1986, 1987, Huang et al 1988, Monteuuis 1994) consists of inserting the excised SAM with a short wedge of underlying tissues into a 2 to 3mm long vertical cut made on the side of the actively growing in vitro raised seedling used as rootstock (Fig 1). In *Sequoiadendron giganteum*, SAM micrografting was more successful on the epicotyl (54%) than on the hypocotyl (21%) (Monteuuis 1987), and likewise in *Pinus pinaster* using 5 to 7 mm shoot apex as scions with 43-50% of grafting success for epicotyl grafts versus 0% for hypocotyl ones (Tranvan and David 1985). In cashew, side-grafting on epicotyl and hypocotyl gave similar grafting success rates (70% and 63% respectively), but hypocotyl-grafted scions elongated faster than when grafted on epicotyl (Mneney and Mantell 2001). Absence of axillary bud on rootstock hypocotyl prevent possible competition between successfully established scions and rootstock shoots, contrary to epicotyl grafts where such “illegitimate” shoots must be cut carefully off (Mneney and Mantell 2001). This remark is applicable to every in vitro grafting technique. SAMs or shoot apices can be also side-grafted between cotyledons, as successfully experienced for *Picea spp* and cultivars (Ponsonby and Mantell 1993), and also for *Pseudostuga menziesii* (Monteuuis 1995a, Fig. 2c). For certain resinous species like *Pinus pinaster*, the wedge of underlying tissues is needless: SAMs are excised at their base by an horizontal cut to be right away placed onto the superficial slash made on the side of the rootstock epicotyl. The exudation flowing out from the parenchyma of the epicotyl further to the removal of the outer tissues enables the early and natural adherence of the grafted SAMs, as depicted in Dumas et al (1989). As for conventional side grafting and contrary to top grafting, once the side micrograft is successfully established, stock main stem must be removed above the grafting point in order to stimulate scion development, and to prevent unsuitable competition with shoots that may arise from the stock.

296 In vitro top-grafting was initially developed by Murashige et al (1972) then Navarro et al
297 (1975) before being successfully transferred to different broadleaved (Alskief and Villemur 1978,
298 Jonard et al 1983, Deogratias at al 1986) and coniferous species (Monteuuis 1994, 1995a). It
299 consists of placing the horizontal cut section of the excised SAM onto the top cut surface of the
300 decapitated epicotyl (Burger 1985) or hypotoctyl (Huang et Millikan 1980) of the in vitro
301 rootstock. On *Citrus spp*, Navarro et al (1975) then Edriss and Burger (1984) observed
302 variations in success rates depending on the location of the scion on the epicotyl section of the
303 decapitated stock. This procedure has been refined for *Citrus spp* by practicing an inverted T
304 incision jut below the epicotyl section of the stock (Navarro et al 1975, Edriss and Burger 1984,
305 Burger 1985, Jonard et al 1987, Navarro 1988). In *Acacia Senegal*, Palma et al (1997) observed
306 at the histological level a nicer graft union when shoot apices were micrografted onto epicotyl
307 compared to hypocotyl.
308 In vitro cleft-grafting seemed the more practised (Mosella et al 1980, Monteuuis 1996,
309 Revilla et al 1996, Danthu et al 2002a and b, 2004, Chabukswar and Deodhar 2006, Wu et al
310 2007): the shoot tip scion is removed with a short wedge of underlying tissues made by two V-
311 shaped cuts at an angle of 20-30°. That will result in an overall scion size ranging from 0.3 mm
312 to 3 cm, depending on the species and on the nature of shoot tips excised i.e. SAMs or bigger
313 shoot apices. As soon as removed, the scion is inserted in the cleft cut made at the top of the
314 decapitated seedling used a rootstock above (Monteuuis 1995a, Fraga et al 2002, Fig 2b, 3a) or
315 below the cotyledons (Corizo et al 2004). Thus, cleft-micrografting 0.5-1cm long shoot tips
316 collected from mature selected *Ziziphus mauritiana* trees onto hypocotyl resulted in more than
317 90% of survival rate and prevented the risks of illegitimate sucker shoot production from the
318 unselected stock encountered with traditional grafting (Danthu et al 2004). Examining the
319 morphological and anatomical aspects of in vitro cleft-grafting of grape cultivars, Cantos et al
320 (1995) noticed that the determining step was to ensure first a good mechanical contact between

rootstock and scion, as for any grafting technique (Hartmann et al 1997). Healing and callus formation began six days after grafting and no subsequent losses were observed further to this initial biological union. The first connections between vascular tissues of rootstock and scions occurred between the 8th and 12th after cleft-grafting, and the grafting union was completed between the 20th and 30th days. .

A comparative study established that side-grafting was more efficient than top-grafting for micrografting SAMs removed from 18-yr old *P. abies* (Monteuuis 1994) and 15 yr-old *Pseudostuga menziesii* individuals (Monteuuis 1995a). This finding was also applicable to the in vitro grafting of 0.4-0.6mm long *Prunus spp* shoot apices (Colin and Verhoyen 1976), of alond (*Prunus dulcis*) cultivars (Yildirim et al 2010), as well as of cashew (*Anacardium occidentale*) individuals (Mneney and Mantell 2001). In contrast, cleft-grafting was more efficient than side-grafting for micrografting shoot apices of *Acacia mangium* (Monteuuis 1996) and of various *Picea spp* ornamental cv (Ponsonby and Mantell 1993).

It could be noted Incidentally that a splice-graft-derived technique (Hartmann et al 1997) was implemented by Gebhardt and Goldba (1988) for studying graft union characteristics in *Prunus sp*, resorting to a piece of translucent silicone tubing for maintaining the scion in tight contact to the rootstock. The use of this micrografting method seems however quite limited.

For top and cleft-grafting more particularly, applying solutions of Diethyldithiocarbamate sodium (DIECA), Polyvinylpyrrolidone (PVP) or ascorbic acid to the scion and to the decapitated section of the stock prior to grafting has been recommended for reducing tissue oxidation in certain fruit tree species (Mosella et al 1980, Jonard et al 1983, 1987). Such treatments were also applied to cashew with variable success (Mneney and Mantell 2001). Inserting a drop of sterilized water or a small block of gelose that could possibly contain growth regulators, nutrients, vitamins and sucrose has been reported to improve in vitro grafting of *Camelia japonica* cultivars (Crézé 1984) and also of various fruit tree species (Jonard et al 1983, 1987,

346 Chimot-Schalll et al 1986), peach (*P. persica*) excluded (Alskief 1977). Notwithstanding that
347 Tranvan and David (1985) used a small bloc of gelose (0.8% of Difco-Agar) mixed with
348 glycerine for in vitro grafting *Pinus pinaster*, applications of exogenous additives to the scion or
349 to the grafting point of the stock prior to micrografting seemed overall less recommendable for
350 coniferous species (Monteuuis 1987, Ponsonby and Mantell 1993). This opinion was shared
351 also by Wu et al (2007) for the in vitro grafting of *Protea cynaroides.*

352 Different kind of devices such as sterilized strips of Parafilm (Danthu et al 2002a, Danthu
353 et al 2004), chromatography tubes (Corizo et al 2004), silicone rings (Revilla et al 1996, Fraga
354 et al 2002) or aluminium foil (Misson and Giot-Wirgot 1985) can be used alternatively for
355 strengthening the connection between the scion and the stock, but such practices have been
356 proven to be needless and impractical for scions of less than 0.5mm in size.

357 Lastly, maintaining the newly micrografted plants under total darkness during 1 to 3
358 weeks just after grafting has been recommended for different species like *Ziziphus mauritiana*
359 (Danthu et al 2002a), *Sequoia sempervirens* (Tranvan et al 1991) and *Picea abies* (Monteuuis
360 1994) where it had been observed to improve significantly SAM micrografting success rates.

361

362 **RECOVERING PATHOGEN-FREE PLANTS**

363 Consistently with Navarro's initial objective (1975), one main interest of in vitro grafting
364 remains to free from viruses and other pathogens valuable *Citrus spp* cultivars (Huang et al
365 1988, Kapari-Isaia et al 2011),. Practically, in vitro grafting of shoot tips has permitted the
366 disease elimination of more than 30 citrus species and cultivars so far (Navarro 1990). The
367 recovery of virus-free in vitro grafted plants depends on the type of virus contaminations, scion
368 size, as well as on the genetic identity of the two symbiotes *i.e.* of the scion and of the rootstock,
369 as demonstrated for citrus (Navarro 1988, Kapari-Isaia et al 2002), and also for grapewine (*Vitis*
370 *vinefera*) by Pathirana and McKenzie (2005). Virus elimination efficiency increases as scion size

371 diminishes, notwithstanding the limitations associated with the manual dexterity of the operator
372 (Murashige et al 1972). The tinier the scion, the greater the frequency of disease-free recovery,
373 but the lower the grafting success (Navarro 1988, 1990). Shoot tip scions of 0.1 to 0.2 mm in
374 length seemed to be the right compromise for ensuring high enough grafting success and virus
375 elimination, which could be improved by green house or in vitro culture preconditioning of the
376 plant material to be grafted Navarro (1990). This view is supported by Deogratias et al (1986)
377 who noticed that the micrografting 0.4 to 1mm long *Prunus avium* apices infested by three
378 different types of viruses resulted in the total recovery of virus-free grafted plants only for tissue-
379 culture-derived apices. For outdoor shoot tips, the rate of disease recovery varied also
380 according to shoot activity*, i.e.* whether it is actively elongating or resting. As developed
381 previously, in vitro pre-culture permit the scions to reach a bigger size than they had at their
382 time of removal from the donor plant, for easier grafting manipulations, hence higher success
383 rates. In peach, Mosella et al (1980) obtained 72% of Shark and 57% of NRS virus-free plants
384 further to the micrografting in controlled conditions of 1cm long pre-treated scions, which were
385 0.4 to 0.8 mm long when they were excised from the donor plants.

386 In vitro grafting has been proven to be very useful also for screening phytoplasma
387 resistance in *Prunus* and *Malus* (Jarausch et al 1999), as well as for viral indexing of
388 grapewines for instance (Pathirana and McKenzie 2005).

389 More generally, in vitro grafting of shoot apices, and SAMs more particularly, can be
390 helpful for initiating contamination-free tissue cultures, and overcoming thereby the disinfection
391 problems associated with the use of bigger primary explants, especially for mature plant
392 materials.

393

394 **OVERCOMING GRAFT INCOMPATIBILITY PROBLEMS**

In vitro grafting has been reported to overcome graft incompatibility problems (Lachaud 1975, Hartman et al 1997) encountered with conventional grafting techniques in various species such as *Ziziphus mauritiana* (Danthu et al 2002a) and *Vitis vinifera* (Pathirana and McKenzie 2005). In vitro micrografting has also permitted to graft successfully peach (*Prunus persica*) onto apricot (*Prunus armeniaca*), which had never been possible in natural conditions due to incompatibility barriers (Martinez et al 1979). Localized incompatibilities, characterized by graft union deficiencies, can be distinguished from more diffuse and time delayed translocated incompatibilities (Lachaud 1975, Jonard 1986). In vitro heterografts were used for studying localized and translocated incompatibilities in various fruit tree species (Jonard et al. 1983), with special mention for *P. cerasifera, P. persica* and *P. armeniaca* (Martinez et al 1979, 1981), which revealed a possible antagonistic effect of a particular peroxydase on grafting (Quessada and Macheix 1984). In vitro grafting has been also useful for detecting precocious diagnostic of grafting incompatibilities between apricot (*P. armeniaca*) cultivars and myrobolan (*P. cerasifera*) rootstocks (Chimot-Schall et al 1986). Micrografting allows deeper investigations on localised incompatibility by examining the quality of the vascular connections at the graft union level with possible resort to radioactive tracers, as developed by Gebhardt and Goldba (1988) on *Prunus.*

In *Acacia mangium*, the micrografting of 0.3 to 0. 4 mm long shoot apices gave similar success rates for young (52%) and mature (46%) donor plants, as against 49 and 0% for the same origins of scions cleft-grafted onto seedlings in nursery conditions (Monteuuis 1995b). The total failure of mature tree grafting was assumed to be caused by localized incompatibility problems, which could arise from histological and biochemical ageing-induced differences (Lachaud 1975). In this regard, the possibility offered by micrografting of miniaturizing scion size to put in contact tissues of similar differentiation degree, irrespective of the age of the donor plant, can solve out localized incompatibility hindrances.

REJUVENATION

Woody plant organogenic capacities, including their ability to be mass propagated true-to-type by rooted cuttings, are closely associated with juvenility (Borchert 1976, Hackett 1985). This juvenile physiological status becomes more and more time and space restricted as the plants increase in size in the course of time, to be ultimately confined to within SAM zones, or even cells, during the period of higher metabolic activity preceding shoot expansion (Krenke 1940, Monteuuis 1989). It can be therefore logically assumed that the more mature the plant, the smaller the portion of SAM tissues liable to contain cells that have remained juvenile at least to a certain extent (Nozeran 1984, Monteuuis 1989). This juvenile potential is being repressed *in situ* by the negative ageing influence of the mature surrounding cells and tissues, these latter becoming more and more important as plants develop (Nozeran 1984). Excising SAMs from such inhibitory correlative systems for placing them in suitable culture conditions has been viewed as a means for allowing the organogenic expression of this within SAM juvenile potential, and for recovering hence the original juvenile physiological status (Nozeran 1984, Monteuuis 1989, Bonga and Von Aderkas 1992). The physiological rejuvenation of a 100-yr-old giant sequoia further to SAM in vitro culture, which has been maintained in culture in a juvenile state ever since, is in this respect quite demonstrative (Bon and Monteuuis 1991, Monteuuis 1991, Monteuuis *et al* 2008). In vitro grafting can be very helpful for achieving this goal considering on the one hand that SAM or shoot apex culture on synthetic media is currently successful only for a limited number of woody species (Bonga et al 2010) , and on the other hand, that in vivo conventional grafting is not compatible with scion miniaturization. Relevantly, with a view to rejuvenate mature selected genotypes of several tree species, efforts have been focused on using as scions the smaller shoot tips that can be micrografted with success, even if quite low. This strategy could be considered far more immediate, straightforward and efficient than long serial grafting procedures, with the unavoidable risks of failure even after several

445 cycles of successive grafting. Like for virus elimination, scions of about 100µm as overall size
446 seem to be the smallest that can be micrografted, bearing in mind that SAMs of a lot of species
447 are much tinier than this (Romberger 1963, Mankessi *et al* 2010). Beside these size constraints,
448 the prediction of the most suitable period for collecting the scions from the mature *in situ*
449 selected individuals is still hazardous. In *Sequoiadendron giganteum*, striking morphological
450 rejuvenations were noticed for some shoots arising from successfully micrografted SAMs
451 derived from 100 yr-old individuals (Monteuuis 1987). 2D PAGE analyses established that
452 scions developing a mature morphological type contained more proteins and mostly acid, than
453 juvenile-like scions (Bon and Monteuuis 1987). Deeper molecular investigations established that
454 the morphologically rejuvenated scions originally derived from 100-yr-old individuals exhibited
455 the J16 protein immunoblot associated with juvenility in *Sequoiadendron giganteum*, but also
456 and more intriguingly, a second band that could be seen only for the materials rejuvenated by
457 SAM micrografting (Bon 1988). This suggests a rejuvenating influence of the juvenile seedlings
458 used as rootstocks itself, including possible transfer of rejuvenating molecules, which seemed in
459 that particular case to have the same recognition site as J 16, but a lightly smaller size. The
460 micrografting of SAMs or shoot apices of less than 0.5mm in length collected from mature trees
461 of species exhibiting noticeable phase change-related foliar dimorphism could give rise to
462 morphological rejuvenations, as illustrated in Fig 4 for *Pinus pinaster*. These morphological
463 rejuvenations were observed to vary a lot in frequency and intensity from one micrograft to
464 another in *Pinus pinaster* (Monteuuis and Dumas 1992) and in *Acacia mangium* (Monteuuis
465 1996) more specifically. The recovery of juvenile foliar characteristics seemed to be associated
466 with the rapidity to resume growth just after grafting (Dumas et al 1989, Cortizo et al 2004). In
467 larch (*Larix decidua*), shoots derived from the in vitro micrografting of SAMs plus underlying
468 tissues (0.3 to 0.5 mm as overall size) removed from 140 year-old selected individual displayed
469 a much higher capacity for vegetative propagation through nodal explants and for adventitious

rooting than shoots collected directly from the same donor trees (Ewald and Kretzschmar 1996). The authors of this work concluded on a rejuvenating influence of micrografting in larch. This opinion was also shared by Perrin et al (1994) on *Hevea brasiliensis* who observed that the micrografting of 1-2 mm long shoot apices onto young seedlings in vitro improved significantly in vitro shoot production and adventitious rooting capacities of the grafted mature clones. Restoration of adventitious rooting competency for shoots issued from micrografting of 0.5 cm long buds collected from mature avocado trees onto young in vitro seedlings constituted another supportive argument of the rejuvenating influence of micrografting (Pliego-Alfaro and Murashige 1987). In *Sequoia sempervirens*, the recovery of juvenile-like traits further to one micrografting of 4-5 mm long shoot tips derived from a 500 yr-old genotype onto in vitro juvenile microshoots varied also substantially in intensity from one micrograft to another (Tanvan et al 1991). These traits included leaf morphology, growth habit with a higher proportion of orthotropic shoots that characterize the juvenile state, and also enhanced capacity for adventitious rooting. However, the more or less pronounced rejuvenations noticed were observed to disappear soon as shoots elongated after transfer to ex-vitro conditions. In *Anarcadium occidentale*, the micrografting of longer long shoot tips (1-2 cm in length) onto young seedlings improved only slightly the competency for adventitious rooting of mature trees, which led Mneney and Mantell (2001) to state that their micrografting method induced only partial physiological rejuvenation in cashew. In citrus, Murashige et al (1972), then Burger (1985) noticed that the plants issued from shoot apex in vitro micrografting bypassed the juvenile phase characterizing nucellar plants. Navarro et al (1975) and Navarro (1988, 1990) further stated that successfully micrografted Citrus plants did not reverse to the juvenile phase. They looked more vigorous than the original contaminated so weaker plants, and also physiologically more mature, bearing fruits much earlier than the nucellar offspring, not always true-to-type (Navarro 1990).

494 Serial or “cascade” grafting has been reported to induce “rejuvenation”, on Douglas fir for
495 instance, as the number of successive grafting cycles of the scions initially collected from
496 mature individuals onto the young seedlings used as rootstocks increases (Franclet 1983).
497 However, it seems more proper to talk about reinvigoration or partial rejuvenation when a
498 regaining of vigor is the main effect resulting from these serial graftings (Borchert 1976, Wareing
499 1987, Pierik 1990). Serial grafting can be applied to in vitro grafting with a view to achieving
500 rejuvenation, which could not be fulfilled by only one grafting operation maybe because of
501 insufficiently small scions. Usually, serial gafting involves bigger scions with more mature
502 tissues left attached below the SAM. The idea is to produce scions with less and less
503 differentiated and mature cells as the number of successive grafting transfers increases. The
504 rejuvenating influence of serial grafting in vitro was extensively studied in *Sequoia sempervirens*
505 using as scions 1.5 cm long shoot tips initially derived from mature individuals, and juvenile
506 microshoots issued from in vitro seedlings as rootstocks (Huang et al 1992a). As the number of
507 micrografting cycles increased, up to four, the authors observed a significant improvement of
508 shoot elongation as well as of the capacities to produce adventitious roots and shoots, to reach
509 similar scores as the juvenile material used as experimental control. Complete restoration of the
510 competency for adventitious rooting was achieved after five repeated cycles of micrografting
511 (Huang et al 1996, Chang et al 2010). This assumption of physiological rejuvenation induced by
512 serial micrografting based on organogenic indicators was strengthened by an increasing
513 number of analogies at the molecular level between with the juvenile controls and the
514 successive generations of serial grafts, and a deeper contrast with the original mature
515 genotypes from which the scions initially derived. These molecular changes included the overall
516 protein pattern (Huang et al 1992a, Chang et al 2010), protein phosphorylation and the
517 expression of particular polypeptides (Kuo et al 1995, Huang et al 2003a, Chang et al 2010),
518 isoesterases and isoperoxidases (Huang et al 1996), as well as mtDNA fragments (Huang et al

519 1995), which could be partly sequenced (Huang et al. 2003b). Also, the rejuvenated shoots
520 issued from these successive cycles of in vitro grafting displayed similar capacity for growth,
521 nitrogen and chlorophyll contents, photosynthesis and respiration rates as the juvenile materials
522 used as control, contrary to what could be observed for the same criteria in the mature original
523 material (Huang et al 2003c). However, all these analyses and findings were restricted to in vitro
524 conditions.

525 In *Olea europea*, Revilla et al (1996) noticed that micrografting 1 to 1.5 cm long shoot
526 tips collected from mature plants gave rise to more vigorous micrografts than when using
527 juvenile in vitro shoots, these latter displaying a slightly higher capacity for micrografting (96-
528 100% vs 64-92% for the mature source scions). The micrografting procedure applied improved
529 noticeably the ability for in vitro adventitious rooting of the mature material (57% vs 2% for non
530 grafted material), which remained lower still than that of the in vitro juvenile shoots (100%). A
531 second cycle of micrografting enhanced a bit more the vigor of the micrografts from the mature
532 source, as well as their ability for micrografting which became similar as for the in vitro juvenile
533 source (100%), but did not improve further the competency for adventitious rooting (48%). The
534 authors concluded on a partial rejuvenation, or more properly on a reinvigoration effect of
535 micrografting on their material. Serial micrografting of 0.5 to 1cm long microshoot tips from
536 mature individuals was also observed to restore the ability for adventitious rooting considered as
537 a rejuvenation indicator after the third generation of repeated grafting for *Faidherbia albida*
538 (Danthu et al 2002b) and *Ziziphus mauritiana* (Danthu et al 2004), and only after the fifth cycle
539 of successive grafting in *Garcinia indica* (Chabukswar and Deodhar 2006). These observations
540 could be interpreted as a rejuvenating effect of serial micrografting, which was also observed to
541 reinvigorate shoot growth for *Faidherbia albida* and *Ziziphus mauritiana*. In *Citrus spp* also
542 seven successive micrografting cycles of 0.2cm long shoot tips derived from mature individual
543 onto young seedlings in vitro improved shoot vigor and adventitious rooting competency (Huang

544 et al 1992b). Conversely, in *Thuja plicata*, serial micrografting of 6 to 7 mm long shoot tips, the
545 smallest scions which could be in vitro grafted, did not result in probative rejuvenating effect
546 (Misson and Giot-Wirgot 1985).

547

548 **CONCLUSIONS**

549 In vitro micrografting combines the advantages of grafting and of in vitro shoot apex and
550 SAM cultures, while overcoming concurrently certain limitations of these to vegetative
551 propagation techniques: incompatibility and persistence of disease problems for the first one,
552 inappropriate protocols for the second one. The time and the skilfulness required as well as a
553 rather low rate of success hinder its utilization at an industrial scale. Like for conventional
554 grafting, in vitro grafting response is prone to vary substantially from one graft to another
555 according to rootstock and scion characteristics, and also to the quality of the union between the
556 two symbiotes. The unavoidable differences associated with the manual manipulations of the
557 operator constitute another factor of heterogeneity. This should not downplay the magnitude of
558 the benefits arising from the positive responses. More efficient vegetative propagation methods
559 can be used for rapidly increasing the number of the too few pathogen-free or rejuvenated in
560 vitro grafts produced. Conjointly more efforts are needed at the basic level for a deeper analysis
561 of the nature of the rootstock influence on the changes induced by in vitro grafting at the scion
562 level, and more generally of the relationships that may exist between genetically and
563 physiologically different tissues and cells.

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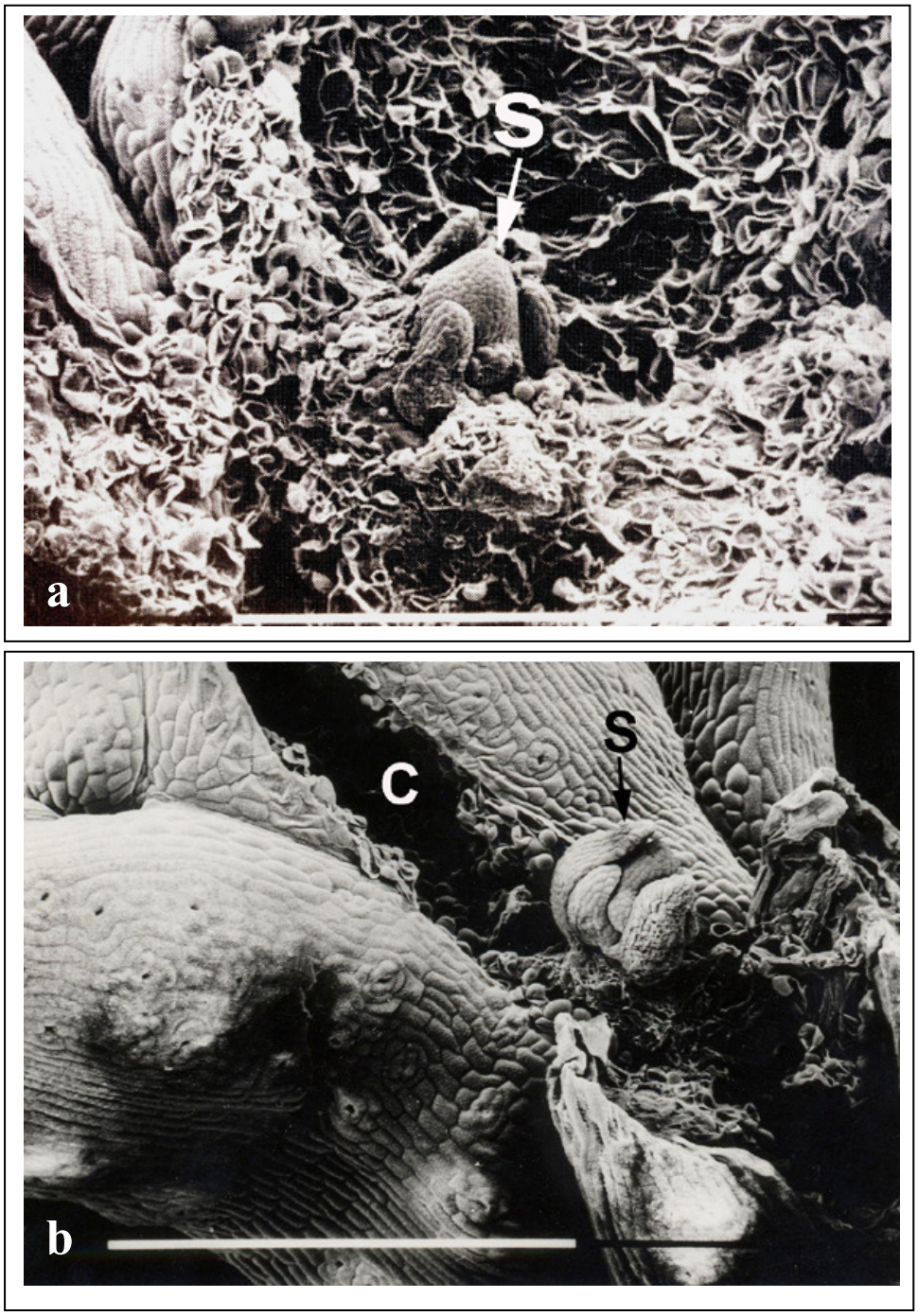

Fig. 1. *Sequoiadrendron giganteum* SAM side-micrografting: a few days after grafting, the leaf initia of the SAM dome grafted (S) have elongated, and the scion is surrounded by a profusion of parenchyma cells actively proliferating from the slit made on the side of the seedling rootstock (a); a more comprehensive view (b) shows the initial side-grafting cut (C) made on the rootstocks and the successfully micrografted SAM (S) with its apical dome surrounded by leaves in their early stages of development (scale bars correspond to 1 mm).

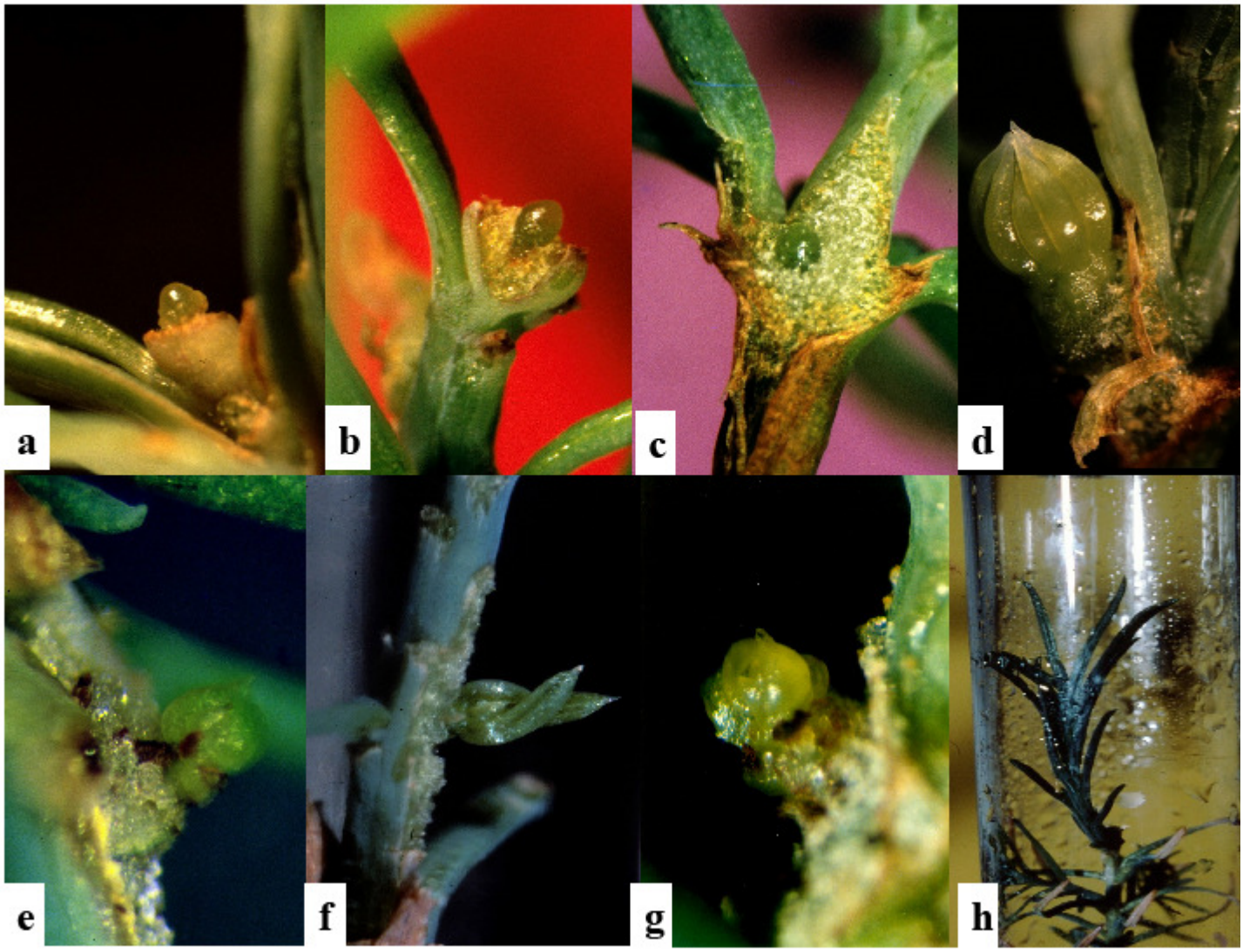

Fig. 2. In vitro SAM “top-grafting” (a), “cleft-grafting” (b), “side-grafting” (c) with a more advanced stage of development (d) of *Pseudotsuga menziesii*, , “side-grafting” (e) with a more advanced stage of development (f) of *Pinus pinaster*, and of *Sequoiadendron giganteum* (g) with the development of a morphologically rejuvenated scion (h).

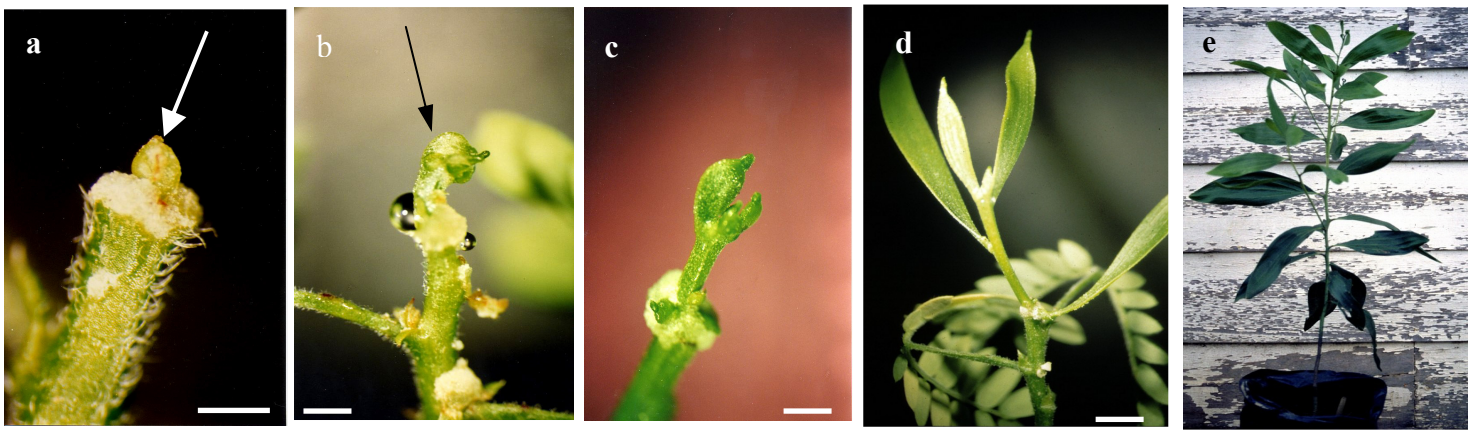

Fig. 3. Shoot apex micrografting of mature *A. mangium*. a) shoot apex (arrow) newly cleft-grafted in axenic conditions onto an in vitro grown seedling rootstock; b), c) and d): scion (arrow) elongating and exhibiting its initial mature-phyllode morphology, by contrast with the juvenile compound leaves produced by the seedling rootstock; e) acclimatized micrograft in outdoor conditions. Scale bars correspond to 1mm for a, b and c, and to 2mm for d..

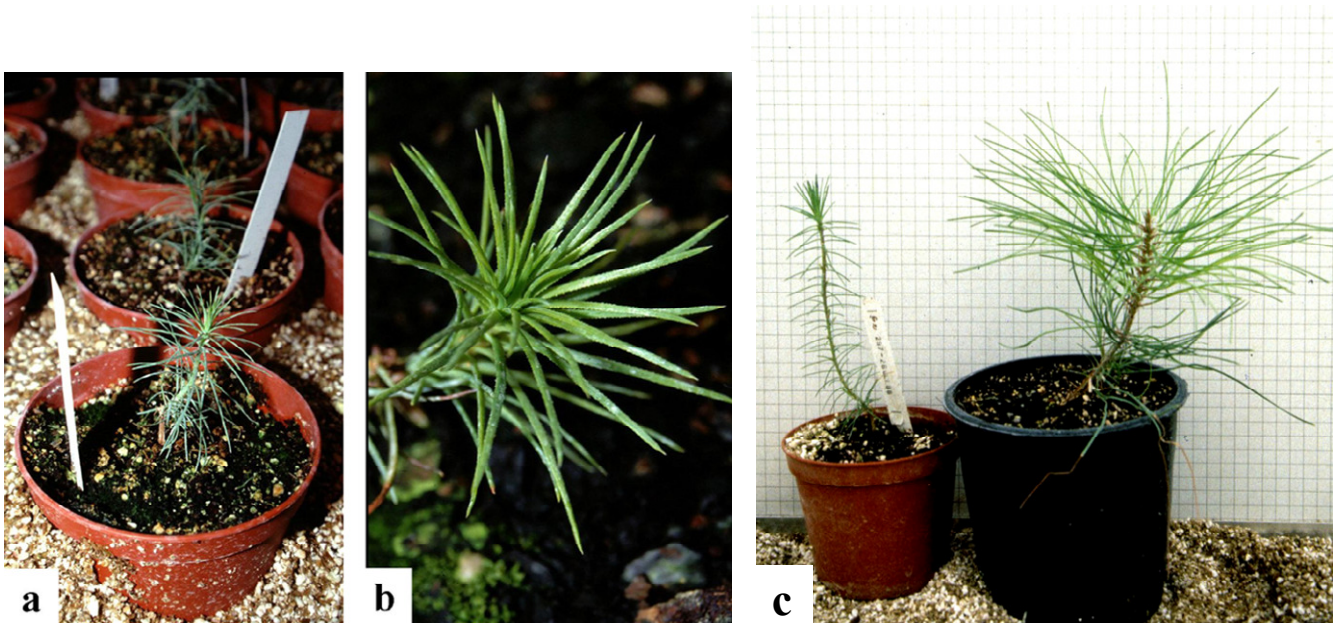

Fig. 4. *In vivo* rejuvenated shoots derived from SAM in vitro micrografting of mature *Pinus pinaster* ortets (a and b) and exhibiting a juvenile morphology compared to a cutting (right) of the same mature ortet that had rooted with difficulty (c).