**THE EFFECTS OF GROWTH REGULATORS ON *IN VITRO* AXILLARY SHOOT PROLIFERATION AND ROOTING OF *ERICA MULTIFLORA***

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**Abstract**

# Multiple shoots were induced on nodal segments of a mature plant of *Erica multiflora* L. Axillary shoots produced on uncontaminated explants were excised, segmented and recultured in the same medium to increase the stock of shoot cultures. The Anderson medium, augmented with different concentrations of 2-isopenthenyladenine (2iP) either singly or in combination with 1-naphthaleneacetic acid (NAA), as potential medium for shoot multiplication by nodal segments was tested. In the following experiment equal molar concentrations of four cytokinins [2iP, kinetin, zeatin and *N*6-benzyladenine (BA)] were tested for ability to induce axillary shoot development from single node stem segments. The highest rate of axillary shoot proliferation was induced on Anderson medium supplemented with, 2.5 g l-1 PhytagelTM and 19.68 μM 2iP. Four indole-3-butyric acid (IBA) concentrations (0, 0.12, 0.24 or 0.49 µM) were tested to determine the optimum conditions for *in vitro* rooting of microshoots. The highest rooting percentages were obtained with IBA at 0.12 and 0.24 μM (72 and 75%, respectively). Eighty percent of the *in vitro* rooted plantlets were successfully established in soil.

**Key words:** *Erica multiflora* L., micropropagation, growth regulators, shoot multiplication, *in vitro* rooting

**INTRODUCTION**

The genus *Erica* (Ericaceae) is represented by approximately 860 species distributed over South Africa, the Mediterranean basin and Western Europe*.* Native *Erica* species are widely distributed in Italy and include *E. arborea, E. carnea, E. cinerea, E. manipuliflora, E. multiflora, E. scoparia, E. sicula,* and *E*. *terminalis.*

*Erica multiflora* is an erect evergreen sclerophyllous shrub that typically occurs in basic soils of the Mediterranean basin. In the island of Sicily the plant is among the components of the coastal shrubland and can be found on calcareous rocky hill slopes and on the edges of woodland; *E. multiflora* vegetative growth occurs twice a year, in spring from February-March to June and in autumn from September to November. Flowering starts in September and continues until November. The flowers are pale pink, narrow and bell-shaped and up to 7 mm in length. Like most Mediterranean native evergreen sclerophylls (Tomaselli, 1981), *E. multiflora* has developed anatomical and physiological adaptations (Margaris, 1981) to tolerate high temperatures and water stress during the hot and dry summer months and therefore has the capacity to perform much better than shrubs introduced from other climatic regions. Although many *Erica* species are cultivated for their attractive ornamental features, *E. multiflora* plants, as far as our knowledge, are not commercially available. However, for its appealing floral display occurring during the fall months and its adaptation to soil and climatic conditions of the Mediterranean region *E. multiflora* could be promoted for use both in landscapes andin the bedding plant industry. In addition, many areas of the Mediterranean basin suffer a progressive process of vegetation disturbance and deterioration as a result of the interaction of various anthropic factors including large scale urbanization and tourism activities (Le Houérou, 1981; Di Pasquale et al., 2004 ; Iapichino et al., 2009). With regard to Sicilian revegetation plans, the incorporation of the native flora has been recently stressed by Raimondo et al. (2009). These authors included *E. multiflora* in a list composed by 173 taxa belonging to 72 genera which were assessed for environmental restoration by literature and field observations. These considerations make it important to focus research on the definition of commercially suitable propagation methods for this unexploited Mediterranean heath. *E. multiflora* multiply naturally by seed and recent findings by Moreira et al. (2010) report 82% germination under laboratory conditions. However, according to these authors *E. multiflora*  initial seedling growth was slow compared to other Mediterranean species tested. Iapichino et al. (2009) in an experiment attempting to reintroduce several native species in the Mediterranean archipelago of the Egadi Islands also recorded slow growth and low rate of survival for *E. multiflora* seedling transplants.

Although, vegetative propagation of many *Erica* species by stem cutting is currently applied (Dunnet, 1987; Hartmann et al., 2003; Dirr and Heuser, 2006), in a preliminary study we found that only 45% of *E. multiflora* softwood stem cuttings, harvested in early fall, root in about 8 weeks. Moreover, propagation by conventional stem cutting may present further disadvantages because implies the constitution of large number of mother stock plants. Nowadays, tissue culture technology is utilized not only in the ornamental industry for large-scale plant multiplication of elite superior varieties (Rout et al., 2006), but also for the conservation and mass propagation of selected native perennial plants (Englemann, 1991; Sudhersan et al., 2003). Micropropagation could be an alternative and effective method for rapid mass propagation of selected clones of *E. multiflora* and would also preserve wild plants from which to take cuttings. Several *Erica* species are commercially micropropagated (Hartmann et al., 2003; McCulloch, 1996), however, specific protocols are restricted to *Erica andevalensis* (Márquez-García et al*.,* 2009*)* and *Erica carnea (*Norton and Norton, 1985*).* Therefore, our objective was to develop a micropropagation system to produce adequate plant material of *E. multiflora* both for ornamental purposes and for eventual reintroduction studies in landscape preservation areas.

**MATERIAL AND METHODS**

***Culture establishment***

Plant material was harvested in October 2009 from wild plants growing at the northern base of Mount Gallo 5 km from Palermo (long. 13° 19' E, lat. 38° 9' N) in the northern coast of Sicily (Italy). Actively growing apical shoot tips, 3-5 cm in length and 0.7-1.0 mm in diameter, and bearing 20-23 nodes, were cut and used as the starting material. All leaves and the apices were removed. Explants were rinsed under running tap water for 10-12 min and then surface-disinfested with a 10-sec immersion in 70% ethanol followed by gentle agitation for 20 min in a bleach solution [1.05% NaOCl (w/v)] containing 20 drops/liter of Tween 20 (Sigma Chemical Co., St. Louis, MO). After being rinsed three times in sterile distilled water, the basal and the apical ends were aseptically removed and explants sectioned in stem segments (0.8-1.0 cm in length) bearing three or five nodes. Individual stem segments were vertically placed into each sterilized 25 x 150-mm test tube containing 10 ml of Anderson basal medium (Anderson, 1984) plus 20 g l-1 sucrose, 2.5 g l-1 PhytagelTM  (Sigma no. P-8169) and 4,92 µM of 2-isopenthenyladenine (2iP) to induce axillary shoot development. After 5 weeks of culture, axillary shoots (≅ 1.5 cm long) developed on primary explants were excised, the terminal buds removed and the resulted stems cut into segments, bearing three or five nodes. Nodal segments were subcultured in the same medium 5 more times at 4 week intervals to increase the stock of shoot cultures. Shoots were then subcultured on the same medium without growth regulators for 1 week before being used either in multiplication treatments or in the rooting treatment. All cultures were maintained at 25°C under a 16-h photoperiod provided by cool-white fluorescent tubes, 30 μmol·m-2·s-1. The same conditions were used for all experiments. This experiment was repeated in October 2010 with new plant material from the same stock plant and performing the same protocol.

***Multiplication protocol***

Randomly selected axillary shoots were cut from stock cultures, the terminal buds removed and the resulted stems cut into segments. Segments comprising an axillary bud and a 5-10-mm internodal stem below the node were transferred to *in vitro* multiplication treatments. In both experiments the medium consisted of Anderson salts, plus 20 g l-1 sucrose, 2.5 g l-1 PhytagelTM (Sigma no. P-8169). All media were adjusted to pH 4.5 with NaOH. After being autoclaved at 121°C at 103 kpa for 20 min, media were dispensed into each sterilized 25 x 150-mm test tube (10 ml medium per tube).

In the first experiment, 2iP concentrations of 0, 4.92, 9.84 or 19.68 µM in combination with 1-naphthaleneacetic acid (NAA) concentrations of 0 and 0.54 μM, were tested in a two-factor, randomized, complete block design with three blocks per treatment and 15 segments per block (total 45 segments). A single explant was cultured per culture tube. In a further experiment, the optimal concentration of growth regulators for inducing axillary shoot proliferation from the first experiment was tested against equal molar concentrations of different cytokinins (zeatin, kinetin, BA). The experiment was a randomized complete block design with three blocks per treatment and 20 segments per block (total 60 shoots). Growth regulators were added to the media prior to autoclaving, except when different cytokinins were compared, growth regulators were filter-sterilized (0.45 μm, Nalgene) and added after the media were autoclaved and cooled to about 50°C. Stem segments were vertically placed into each test tube. For all multiplication treatments, nondestructive observations were performed after 4 and/or 8 weeks cultureandshoot proliferation score were based on the number of usable shoots (*>* 5 mm long). In both experiments, Fisher’s protected least-significant-difference at (P ≤ 0.01)was used to separate the means for significant effects from the analysis of variance (Petersen, 1985).

# ***In vitro rooting and plant greenhouse establishment***

# Microshoots ≅ 1.5 cm long with intact apex were trimmed of basal leaves and transferred to the root induction media. To determine the optimum conditions for *in vitro* rooting, media containing Anderson salts, 20 g l-1 sucrose, 2.5 g l-1 PhytagelTM (Sigma no. P-8169)were dispensed into sterilized 25 x 150-mm test tube (10 ml medium per tube). Four indole-3-butirric acid (IBA) concentrations (0, 0.12, 0.24 or 0.49 µM) were tested in a randomized complete block design with 4 blocks per treatment and 20 shoots per block (total 80 shoots). A replication consisted of a single explant placed into each test tube. After 5 weeks, data were recorded as percentage of shoots rooted, number of roots per shoot and mean root length. Percentage data were subjected to arcsin transformation before ANOVA analysis. Mean separation was performed by Fisher’s protected least-significant-difference test (*P* ≤ 0.01) (Petersen, 1985). *In vitro* rooted plantlets were removed from culture media and PhytagelTM was washed from the roots. In April 2010, plantlets were transplanted into trays containing a peat-perlite mixture 1:1 (v/v) and covered with clear plastic bags to maintain a high relative humidity. The growing mixture was kept moist by frequent watering and trays were kept in an unheated greenhouse covered with clear polyethylene plus 70% shadecloth. Greenhouse temperatures during the acclimation period were 12-14 °C during the night and 16-22 during the day. Ventilation of the plantlets was increased with time by increasing size of the holes made in the plastic, and after 3 weeks the plastic was removed. After 5 weeks, acclimatized single plants were transferred into 12-cm plastic pots containing the same growing mixture. Plants were submitted to phenotypic evaluation.

### RESULTS AND DISCUSSION

***Culture establishment***

Visual screening showed that 70% of the primary explants appeared free of fungal or bacterial contamination after the disinfenction procedures described in this study were used. Axillary buds started to grow from nodal explants within two weeks in medium supplemented with 4.92 μM 2iP and were about 6.0 mm tall after 4 weeks. Shoots subcultured onto the same medium continued to proliferate new axillary shoots. Transferring single shoots into the same medium without growth regulators resulted in main shoot growth, with no further axillary shoot proliferation.

***Multiplication***

Shoot proliferation occurred through development of existing axillary buds. The effects of NAA and 2-iP concentrations on axillary shoot development after four and eight week culture are shown in Table 1. The presence of the auxin NAA in the medium reduced axillary shoot proliferation. In fact, at all 2iP levels, shoot multiplication was significantly lower with NAA concentration of 0.54 μM than at 0 μM. This is consistent with the findings of Gebhardt and Friedrich (1987) who reported that another auxin, indole-3-butyric acid (IBA) at 10 mg l-1 in combination with the cytokinin BA at 0.1 mg l-1 reduced the number of shoots per explants in *Calluna vulgaris*, a related species belonging to the Ericaceae. After 4 weeks culture, we obtained a maximum of 3.5 shoots per nodal segment in the medium supplemented with 19.68 μM 2-iP and 0 μM NAA. However, the effectiveness of this treatment did not differed significantly from those with lower 2iP concentrations. After eight weeks in culture, regardless of NAA concentration, the number of shoots per explants significantly increased when the medium contained 2iP at 19.68 μM compared to 4.92 and 9.84 μM 2iP. The NAA concentration x 2iP concentration interaction was significant. The highest shoot multiplication (5.2 shoots per explant) was obtained in combination of 0 μM NAA and 19.68 μM 2iP (Fig. 1A). This results suggest that growth regulator requirements for shoot proliferation in *E. multiflora* can be satisfied by 2iP alone. The best result in terms of shoot length, after 8 weeks culture, was obtained from the medium without growth regulators. Shoots produced on the medium supplemented with 0 NAA μM and 19.68 μM 2iP attained growth sufficient (10.6 mm) for the transfer to root induction medium. The combination of 0 NAA μM and 19.68 μM 2iP produced favourable results in terms of number and length of axillary shoots produced and, therefore, was used in equal molar comparisons with other cytokinins (zeatin, BA, and kinetin). Among the cytokinins tested, 2ip and zeatin were more effective than kinetin and BA in promoting axillary shoot proliferation (Table 2). However, zeatin’s higher cost make it a less suitable cytokinin for *E. multiflora axillary* shoot induction. Shoots proliferated in the medium supplemented with 2iP were significantly longer (11.3 mm) than those produced from node explants exposed to other cytokinins.

***In vitro rooting and plant greenhouse establishment***

*In vitro* rooting of excised shoots was accomplished in about five weeks (Fig. 1B). Rooting in absence of growth regulator resulted in only 35% success. The highest rooting percentages were obtained with IBA at 0.12 and 0.24 μM (72 and 75%, respectively) (Table 3). Exposing microshoots to IBA at 0.49 μM substantially reduced rooting performance (51% rooting). Eighty percent of the *in vitro* rooted plantlets transferred to soil (Fig. 1C) survived regardless of the medium tested to induce rooting (data not shown). A total of 180 plants were established in the greenhouse.

Conclusions???

The work presented in this paper is the first report of successful micropropagation of *E. multiflora* through nodal explants from mature plants. The node culture method has been proven to be convenient and reliable to micropropagate several Mediterranean shrubs including *Myrtus communis* (Ruffoni et al., 1994; Scarpa et al., 2000), *Arbutus unedo* (Mereti et al., 2002), *Thymus capitatus* (Iapichino and Bertolino, 2011), *Cistus* spp. (Iriondo et al., 1995), *Rosmarinus officinalis* (Misra and Chaturvedi, 1984), *Lavandula stoechas* (Nobre, 1996), *Crataegus monogyna* (Iapichino and Airò, 2009), and *Lithodora rosmarinifolia* (Iapichino, 2007). In our study, *E. multiflora* shoot tips collected during the fall, corresponding to a period of an active growth phase, gave a good *in vitro* plant establishment response. The highest rate of axillary shoot proliferation was induced on Anderson phytagel medium augmented with 19.69 μM 2iP. Explants were divided, subcultured and continued to proliferate shoots. It was estimated a proliferation rate of 5.2 shoots per single node explants every eight weeks. The *in vitro* rooting and acclimatization process took approximately 13 weeks. The regenerated plants were morphologically normal and started to flower in Fall 2011 (Fig. 1D). Therefore, the *in vitro* propagation technique can be applied as an alternative method of propagation to exploit *E. multiflora* for revegetation studies and ornamental purposes.

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**Legend to Figure**

Figure 1. Micropropagation of *Erica multiflora* through shoot multiplication. A) Cluster of axillary shoots proliferated from one-node stem segment cultured in Anderson medium with 19.69 μM 2iP after 8 weeks culture. B) Plantlet with *in vitro*-regenerated root system. C) *In vitro* regenerated plant 3 weeks after transfer to soil. D) Flowering plant.