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Propagation of *Ptilostemon chamaepeuce* (L.) Less through tissue culture

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Ptilostemon chamaepeuce, a plant native to Crete, has the potential to develop into a new floricultural crop; therefore regeneration *via* organogenesis was studied. Seeds were germinated at rates higher than 84% to produce plant material for *in vitro* culture. The influence of growth regulators was investigated on MS and WPM media supplemented with 0, 1, 2 or 4 mg l^{-1} BA and 0, 0.5, 1, or 2 mg l^{-1} NAA and their combinations. BA at high concentrations (2 or 4 mg l^{-1}) increased the number of shoots (7.6 shoots per explant), while longer shoots were recorded in media without growth regulators. Shoot proliferation was similar in both MS and WPM media. However, chlorosis and dryness of leaves were observed on WPM. Regenerated shoots rooted after culture in MS medium with 0.5, 1 or 2 mg l^{-1} NAA and 1, 2, or 4 mg l^{-1} IBA at rates higher than 80%. No rooting was observed in media without auxins. Rooted cuttings were easily acclimatized (90% survival) in polystyrene plugs at low intensity light and were successfully established in pots under greenhouse conditions.

Key words: *in vitro* culture, new ornamental plant, *Ptilostemon chamaepeuse*, seedling.

INTRODUCTION

Ptilostemon chamaepeuce, which is grown in the eastern Mediterranean countries as well as in Crete, has the potential to develop into a new floricultural crop, mainly for xeriscape landscapes. The use of native plants in local parks and gardens contributes to high biodiversity and sustainability and emphasizes the traditional character of the area of origin (Diekelmann & Schuster, 2002). *P. chamaepeuce,* in its natural habitat, is generally propagated by seed, producing plant communities with great uniformity in morphological characteristics. Seeds collected in Crete are well developed (over 14 mg in weight) and germinate at temperatures between 10 and 20˚C (Antonidaki-Giatromanolaki, 2006). Rooting of stem cuttings of *P. chamaepeuce*, depends on season, substrate, applied auxins etc. and is generally difficult (Antonidaki-Giatromanolaki *et al*., 2008). Moreover, there are quantities of plant material not enough to be used for mass propagation.

Micropropagation exhibits advantages compared to conventional vegetative propagation since it is more effective, faster and results in disease free plants (George, 1996; Harbage, 2001). So far, there are no reports on successful *in vitro* propagation of *P. chamaepeuce* using vegetative explants a fact probably due to the presence of a thick indumentum (Chimonidou *et al*., 2005; Antonidaki-Giatromanolaki, 2006). Many researchers have used explants from seedlings to initiate adventitious shoot or root formation for *in vitro* culture of various species (Schroeder & Stimart, 1999; Choffe *et al*., 2000; Li *et al.*, 2000; Vlahos & Dragassaki, 2000; Harbage, 2001; Hatzilazarou *et al*., 2001; Gutierrez-Nicolas *et al*., 2008).

Propagation of some woody plants has been found to be more successful on media with lower levels of

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nitrogen such as the Woody Plant Medium (McCown & Lloyd, 1981) than on the MS medium (Romano, 1992). WPM contains on average more PO_4^{3-}, SO_4^{2-} and Mg^{2+}, but less NO_3^- and NH_4^+ compared to the media used for herbaceous plants (George, 1996). In the present study we used both MS and WPM media in order to decide on the best medium for shoot proliferation of *P. chamaepeuce.* The experiment was conducted with explants derived from seedlings that had grown from sterilized seeds of the same plant, in order to keep as much as possible, the uniformity of the genetic material. The aim of the study was to establish a protocol for *in vitro* mass propagation of *P. chamaepeuce*, in order to obtain propagation material for commercial use.

MATERIAL AND METHODS

Seed sterilization and germination conditions

Nodal explants of *P. chamaepeuce* from greenhouse mother plants, failed to be established *in vitro*, due to severe contamination or damage. Thus, seeds were collected from one wild individual plant of *P. chamaepeuce* in July 2004 and got dried for 30 days in open air at room temperature. They were then stored in an opaque plastic container at room temperature until their use. Seeds with a weight over 14 mg were washed with tap water and house detergent and then immersed in 0% (sterile water), 70% or 95% ethanol for 15 sec. They were subsequently disinfected in 2.5%, 5% or 10% CaO_2Cl_2 solutions (containing 1 drop of Tween 20 per 100 ml solution) for 10 min and rinsed three times with deionized sterilized water. Afterwards, seeds were placed in test tubes containing a half strength MS medium (Murashige & Skoog, 1962) (Sigma Chemical Co., Germany) supplemented with 8 g l^{-1} agar (Fluca, Biochemika, Germany). The pH was adjusted to 6.0. All steps were carried out under aseptic conditions in a laminar hood. Seeds were kept in a growth chamber at 20 ± 1 °C and 16/8 h photoperiod using 36 μmol $m^{-2}s^{-1}$ white fluorescent tubes (Philips TLT, 36 W).

Five seeds were placed into each test tube (200 × 28 mm) containing ca. 15 ml of the medium and 5 tubes were used per treatment. The experiment was repeated three times. The percentage germination data were transformed into angular values before statistical analysis. The statistical analysis of the data was based on analysis of variance and the means were compared using Tukey's test.

Establishment phase

Shoot tips (approx. 0.5 cm in length) of 2-week old *in vitro* grown seedlings (Fig. 1A) were excised and cultured on a full strength MS medium supplemented with 0.5 mg l^{-1} BA and 30 g l^{-1} sucrose, with pH 5.8. After three subcultures and when a sufficient number of shoots had been obtained, the tips of these shoots, (containing the apical bud plus 2-3 axillary buds) were used as explants for *in vitro* culture.

Multiplication phase

Explants from the previous phase were cultured on MS or WPM media (McCown & Lloyd, 1981) (amended with 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine-HCl and 0.1 mg l^{-1} thiamine-HCl) both supplemented with 0, 1, 2 or 4 mg l^{-1} BA and 0, 0.5, 1 or 2 mg l^{-1} NAA in all possible combinations. Explants were individually embedded into test tubes (100 × 28 mm) containing approx. 12 ml of the medium. Twenty tubes (replications) were used per treatment in a completely randomized experimental design. Cultures were incubated as described above. After 5 weeks in culture, the percentage of shoot formation, number of shoots and the mean length of shoots per explant were assessed. The data were subjected to analysis of variance and the means were compared using Tukey' test.

Rooting phase

Shoots from seedlings grown in MS medium as described above, were cultured on MS medium (Sigma Chemical Co) supplemented with 0.5, 1 or 2 mg l^{-1} NAA or 1, 2 or 4 mg l^{-1} IBA. A control treatment without growth regulators was also included. One explant was introduced into each test tube. Twelve explants were used per treatment in a completely randomized experimental design. Five weeks after the initiation of the culture, the rooting percentage, the number of roots per micro-shoot and the mean length of roots per micro-cutting, were recorded. The experiment was repeated twice and the percentages of rooting were transformed into arcin before statistical analysis of variance.

Acclimatization phase

After five weeks in rooting medium, 30 rooted microcuttings were transferred to polystyrene microplugs and maintained in a growth chamber at 24 ± 2 °C under 16/8h photoperiod (36 μmol m^{-2} s^{-1} cool-white

fluorescent lamps). They were moistened with 10% of Hoagland's nutrient solution in deionized water twice a week. After two months, they were transferred to the greenhouse in pots (9 cm diameter) containing compost (Florabella, Klasman): perlite (4:1 v/v).

RESULTS AND DISCUSSION

Seed sterilization and germination conditions

Results from seed sterilization using ethanol indicated that no germination occurred. Moreover, the seeds treated with ethanol turned dark green to black and decayed. By contrast, a high germination percentage (84-96%) was recorded in all treatments disinfected with CaO_2Cl_2 solution without the application of ethanol (Table 1). Although the germination percentage in the control (sterile water) was high (100%), in the uninfected seeds it was low (20%) and relatively high in the seeds disinfected with CaO_2Cl_2 (79-91%). This experiment was repeated three times with different duration of the ethanol exposure and the results were similar (data not shown). Thus, the application of CaO_2Cl_2 without using ethanol, was the only sufficient method for sterilization of *P. chamaepeuce* seeds.

Establishment phase

Shoot tips, which were excised from *in vitro* germinated seeds, were cultured *in vitro* on MS nutrient medium without any sign of infection (data not shown). During subculturing of these explants *in vitro*, no further problems or chlorosis were observed.

Multiplication phase

Explants in BA and in a combination of BA/NAA responded well and formed shoots by 70% to 100% in MS medium and by 50-100% in WPM medium. By contrast, explants in NAA alone or in media without growth regulators (control) had formed no new shoots except for those from the initial shoots (Table 2).

Two weeks after culture initiation on MS medium, adventitious shoots began to appear at the base of the explants in all treatments except for the control and in all NAA concentrations, without any BA addition. After five weeks of incubation, most of the treatments produced adventitious shoots to a varying degree (Figs 1B, 1E). Best results (7.6 shoots per explant) were obtained on MS supplemented with BA/NAA at a ratio of 2/0, without exhibiting statistical differences from the treatments BA/NAA at 4/0, 4/1, 4/0.5, 2/0.5 and 1/0 (Table 2). The addition of BA increased significantly the number of shoots, particularly without NAA. Increasing of the concentration of NAA with BA, tended to reduce the number of shoots, except for the highest concentration of BA. The shoots grew longer on MS media lacking growth regulators and followed in size by the treatment 0.5 mg l^{-1} NAA (Table 2). Addition of NAA to all concentrations reduced shoot growth compared to the control and the combined treatments with BA and NAA.

At high BA concentrations, more adventitious shoots were induced, originating from the callus and the leaf axils. When the treatments contained only BA (no NAA), more shoots were developed on the axils of explants than on the adventitious shoots at their base (data not shown). Furthermore in the com-

TABLE 1. Effect of different sterilization method on the *P. chamaepeuce* seed germination *in vitro*[1]. Values followed by the same letter are not significantly different ($P \leq 0.05$)

Method of sterilization	Germination %	Not infected seeds (% of the germinated seeds)
control (sterile water)	100[a]	20[b]
95% ethanol	0[b]	–
95% ethanol + 2.5% CaO_2Cl_2	0[b]	–
70% ethanol + 5% CaO_2Cl_2	0[b]	–
70% ethanol + 10% CaO_2Cl_2	0[b]	–
2.5% CaO_2Cl_2	96[a]	79[a]
5% CaO_2Cl_2	92[a]	86[a]
10% CaO_2Cl_2	84[a]	91[a]

[1] 25 seeds per treatment

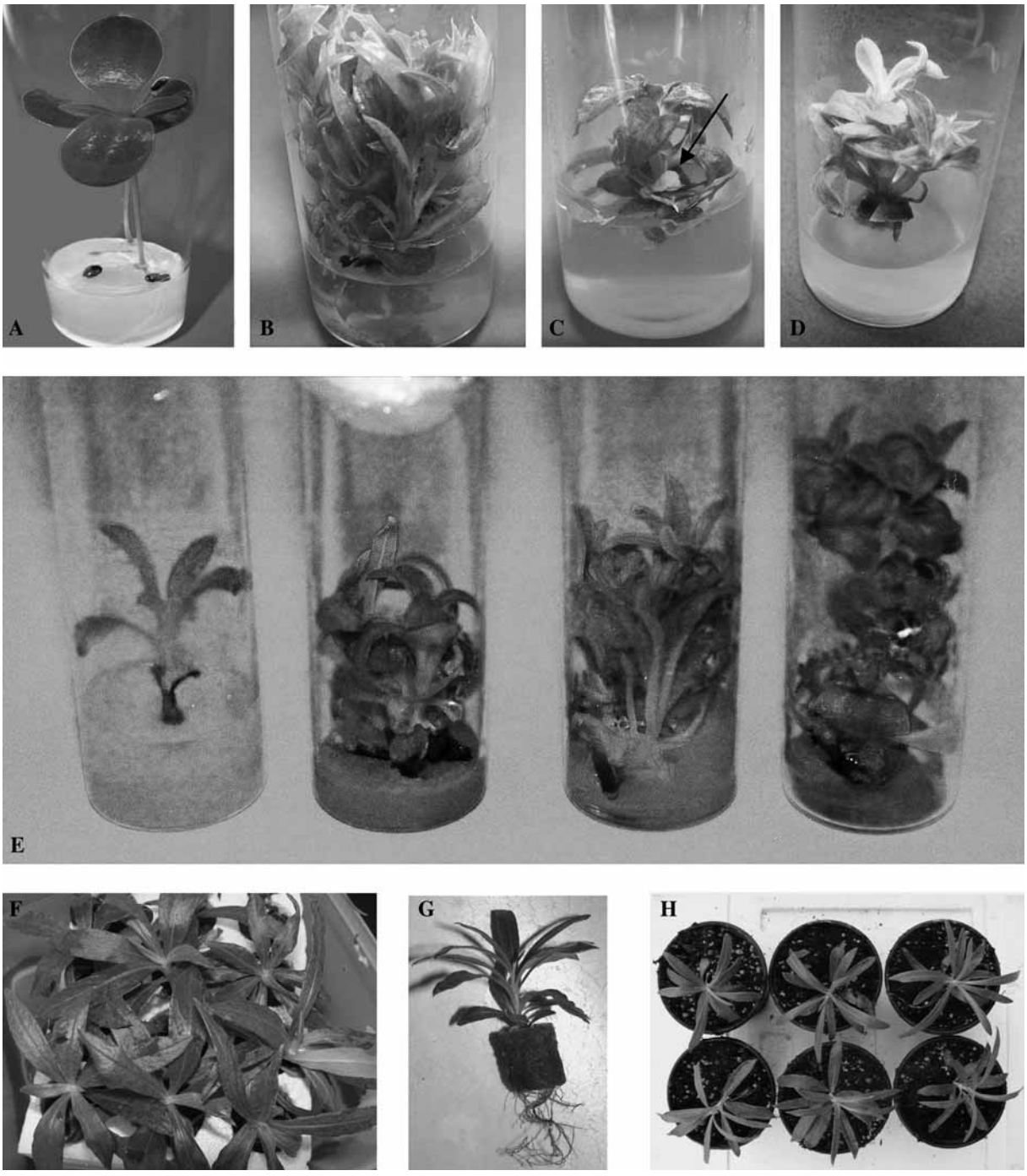

FIG. 1. Micropropagation of *Ptilostemon chamaepeuce* from seedlings. (A) Seedling in half strength MS medium. (B) Adventitious shoot formation on MS medium at 4/1 mg l^{-1} BA/NAA. (C) Hard callus formation (arrow) at the base and the nodes of the explants at high concentrations of BA (4 mg l^{-1}). (D) *In vitro* shoot formation from tip explants on WPM medium. Chlorotic and dried tips of leaves and shoots were developed three weeks after the initiation. (E) Adventitious and axillary shoot formation from explants derived from seedling shoot tips on MS medium at 0/0, 2/0.5, 4/1 and 4/0 mg l^{-1} BA/NAA after five weeks of incubation (from left to right). (F, G) *P. chamaepeuce* rooted microcuttings in plugs with rock wool after 4 (G) and 8 (F) weeks. (H) Plants grown *in vitro* after transplanting to pots under greenhouse conditions.

TABLE 2. Effect of BA, NAA and nutrient media (MS or WPM) on the shoot formation (%), number and length of shoots of *P. chamaepeuce*[1, 2]. Values within column followed by the same letter are not significantly different ($P \leq 0.05$) by Tukey's test

BA/NAA (mg l^{-1})	MS			WPM		
	Shoot formation (%)	Number of shoots	Length of shoots (mm)	Shoot formation (%)	Number of shoots	Length of shoots (mm)
0/0	25^{a}	1.3^{ab}	10.1^{e}	10^{a}	1^{ab}	4.5^{a-e}
0/0.5	10^{a}	1.1^{a}	8.5^{de}	5^{a}	0.5^{a}	2.5^{a-c}
0/1	25^{a}	1.4^{ab}	6.6^{cd}	5^{a}	0.7^{ab}	2.2^{ab}
0/2	15^{a}	1.2^{a}	3.8^{a-c}	5^{a}	0.7^{ab}	1.7^{a}
1/0	100^{c}	5.8^{d-f}	5.5^{a-d}	90^{cd}	4^{cde}	5.2^{a-e}
1/0.5	100^{c}	4.1^{cd}	4.1^{a-c}	50^{b}	3^{b-e}	4.6^{a-e}
1/1	95^{bc}	4.9^{c-e}	4.1^{a-c}	70^{b-d}	2.2^{a-d}	6.2^{de}
1/2	90^{bc}	3.9^{bc}	4.5^{a-c}	60^{bc}	1.9^{a-c}	2^{ab}
2/0	100^{c}	7.6^{f}	5.1^{a-c}	100^{d}	4.5^{de}	6.6^{e}
2/0.5	95^{bc}	5.9^{d-f}	5.2^{bc}	80^{b-d}	2.5^{a-d}	6.1^{c-e}
2/1	95^{bc}	3.6^{a-d}	3.6^{a-c}	60^{bc}	2.6^{a-d}	7.2^{e}
2/2	70^{b}	2.4^{a-d}	4.1^{a-c}	80^{b-d}	3^{b-e}	4.8^{a-e}
4/0	100^{c}	7.5^{ef}	5^{a-c}	100^{d}	4.9^{e}	5.4^{b-e}
4/0.5	100^{c}	5.6^{d-f}	4.7^{a-c}	100^{d}	4.5^{de}	5.3^{a-e}
4/1	100^{c}	7.2^{ef}	2.9^{ab}	80^{b-d}	2.9^{b-e}	4.2^{a-e}
4/2	100^{c}	4.8^{c-e}	1.7^{a}	50^{b}	0.5^{a}	2.7^{a-d}

[1] 20 explants per treatment

[2] Explants were kept at 24 ± 2 °C and 16h/8h photoperiod for 5 weeks

binations of BA and NAA, more adventitious shoots were formed at the base of the shoots (Fig. 1E). In comparison to other treatments, callus formation was observed in all treatments with NAA and in the combinations of BA and NAA. The callus was soft and friable in the explants of the NAA treatments and hard in the explants of the combinations of BA and NAA. Hard callus was also formed at the base and at the nodes of the explants, mostly at high BA concentrations (Fig. 1C), which produced shoots after 2 or 3 subcultures. Vitrification was recorded at high BA and BA/NAA concentrations when the explants were cultured for more than five weeks.

Cultures in WPM medium supplemented with BA only or combination with BA and NAA stimulated shoot proliferation compared to the control or the NAA alone (i.e. no BA) treatments (Table 2). The effect of BA and NAA on shoot proliferation was comparable to that in the MS medium, except for the fact that less shoots were formed per explant. The higher the concentration of BA, the greater the mean number of shoots produced per culture, after 5 weeks of incubation. NAA had an inhibitory effect on shoot proliferation in cultures with BA. Three weeks after the initiation of the culture on WPM medium, the leaves were chlorotic. Furthermore, dryness of leaf tips and shoots in all treatments was recorded (Fig. 1D). The most serious symptoms of dryness were observed in the explants treated with high concentrations of BA and NAA combinations (BA/NAA: 4/2 mg l^{-1}).

The fact that MS medium had better results than WPM on *in vitro* proliferation and shoot induction of *P. chamaepeuce*, has also been mentioned by other researchers in *Pitosporum napaulensis* (Dhar *et al*., 2000) and *Sterculia urens* (Hussain *et al*., 2008).

Root formation was observed in cultures with NAA alone in both media, and the first roots were visible from the second week after cultivation in the

TABLE 3. Effect of NAA and IBA concentrations on *in vitro* rooting of *P. chamaepeuce* microshoots five weeks after planting[1,2]. Values within column followed by the same letter are not significantly different ($P \leq 0.05$) by Tukey's test

Treatment	Concentration (mg l^{-1})	Rooting (%)	Mean number of roots/shoot	Mean length of roots/shoot (cm)
Control	0.0	0.0^{a}	0^{a}	0^{a}
NAA	0.5	41.7^{b}	4.8^{ab}	2.9^{ab}
	1.0	44.4^{b}	4.6^{ab}	2.8^{ab}
	2.0	83.3^{c}	11.9^{b}	6.3^{ab}
IBA	1.0	50.0^{bc}	4.5^{ab}	3.5^{ab}
	2.0	50.0^{bc}	8.7^{ab}	6.8^{b}
	4.0	75.0^{bc}	14.2^{b}	6.4^{ab}

[1] 12 explants per treatment
[2] Explants were kept at 24 ± 2˚C and 16h/8h photoperiod for 5 weeks

rooting media. No explants were rooted from the control and from the treatments containing BA alone or in combination with NAA.

Rooting phase

Ten days after the initiation of the culture, the first roots were visible from the formed callus at the base of some explants. In five weeks, the root system had been satisfactorily developed. None of the explants rooted in a medium without auxins (Table 3). The best rooting percentage was recorded at 2 mg l^{-1} NAA (83.3%), followed by the treatment at 4 mg l^{-1} IBA (75%). However no statistical differences were recorded between all IBA treatments and the 2 mg l^{-1} NAA and also among treatments concerning root number and root length (Table 3). Although rooting of stem cuttings *ex vitro* is difficult (Antonidaki-Giatromanolaki *et al*., 2008), rooting of microshoots is easier by using the MS medium supplemented with NAA or IBA at the above mentioned concentrations. Moreover, IBA and NAA have been reported to have a stimulatory effect on root induction in many species including *Pitosporum napaulensis* (Dhar *et al*., 2000), *Bambusa vulgaris* (Ndiaye *et al*., 2006), *Sterculia urens* (Hussain *et al*. 2008) and *Tectona grantis* (Akram & Afrab, 2008).

Acclimatization phase

Ninety percent of the rooted microcuttings were successfully acclimatized and were transferred two months later to the greenhouse in pots (9 cm in diameter) containing compost (Florabella, Klasman): perlite (4:1 v/v). All rooted micro-cuttings, which had been planted first in the polystyrene microplugs, were successfully established later in the greenhouse (Figs 1F, G and H). By contrast, rooted microcuttings planted in pots and transferred directly to the greenhouse had a very low percentage of establishment (data not shown).

CONCLUSIONS

This paper reflects the first attempt to micropropagate *P. chamaepeuce* by tissue culture. Using the typical protocol for seed disinfection, we ascertained that ethanol seriously harmed seeds, which did not germinate. The results on shoot proliferation showed that *P. chamaepeuce* can be propagated *in vitro*, using shoot tips obtained from seedlings producing up to 7.6 shoots per explant in five weeks. Explants in MS medium produced more and longer shoots with better quality than in WPM. The percentage of rooting of *P. chamaepeuce* microcuttings was higher than 80% at 2 mg l^{-1} NAA and 75% at 4 mg l^{-1} IBA and the young plantlets established easily in pots under greenhouse conditions with a survival rate higher than 90%.

REFERENCES

Akram M, Afrab F, 2008. High frequency multiple shoot formation from nodal explants of teak (*Tectona grandis* L.) induced by thiadizuron. *Propagation of ornamental plants*, 8: 72-75.

Antonidaki-Giatromanolaki A, 2006. *Development of native species of Crete in the Urban landscape: an investigation of systems and plant reproduction, adaptability and fitness of wild species in commercial horticulture.* Ph.D. Thesis. Natural Resource Institute, University of Greenwich, Chatham U.K.

Antonidaki-Giatromanolaki A, Orchard J, Papadimitriou M, Dragassaki M, Vlahos I, 2008. Propagation of stem cuttings of *Ptilostemon chamaepeuce* (L.) Less. *Propagation of ornamental plants*, 8: 186-189.

Chimonidou D, Vlahos JC, Odyseos E, Georgiou K, Della A, 2005. Evaluation of species from Cyprus flora for sustainable use in commercial floriculture. *Acta horticulturae*, 683: 111-119.

Choffe KL, Murch SJ, Saxena PK, 2000. Regeneration of *Echinacea purpurea*: Induction of root organogenesis from hypocotyls and cotyledon explants. *Plant cell tissue and organ culture*, 62: 227-234.

Dhar U, Upreti J, Bhatt ID, 2000. Micropropagation of *Pittosporum napaulensis* (DC.) Rehder & Wilson – a rare, endemic Himalayan medicinal tree. *Plant cell, tissue and organ culture*, 63: 231-235.

Diekelmann J, Schuster R, 2002. Natural landscaping: *Designing with native plant communities*. Second ed., The University of Wisconsin Press, Madison, WI.

George EF, 1996. Plant propagation by tissue culture, Part 2, In Practice, 2nd (revised) Edition. Exegetics Ltd., Edington, Wilts, England.

Gutierrez-Nicolas F, Ravelo AG, Zarate R, 2008. Seed germination and in vitro propagation of *Maytenus canariensis* through regeneration of adventitious shoots from axillary and apical buds. *Biologia plantarum*, 52: 173-176.

Harbage JF, 2001. Micropropagation of *Echinacea angustifolia*, *E. pallida*, and *E. purpurea* from stem and seed explants. *Hortscience*, 36: 360-364.

Hatzilazarou S, Economou A, Antoniou T, Ralli P, 2001. Propagation of *Ebenus cretica* by tissue culture. *Propagation of ornamental plants*, 1: 25-27.

Hussain TM, Chandrasekhar T, Gopal GR, 2008. Micropropagation of *Sterculia urens* Roxb., an endangered tree species from intact seedlings. *African journal of biotechnology*, 7: 95-101.

Li H, Murch H, Saxena PK, 2000. Thidiazuron-induced *de novo* shoot organogenesis on seedlings, etiolated hupocotyls and stem segments of Huang-qin. *Plant cell tissue and organ culture*, 62: 169-173.

McCown BH, Lloyd DG, 1981. Woody plant medium (WPM). A mineral nutrient formulation for microculture of woody plant species. *Hortscience*, 16: 453.

Murashige T, Skoog F, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia plantarum*, 28: 473-497.

Ndiaye A, Diallo MS, Niang D, Gassama-Dia YK, 2006. *In vitro* regeneration of adult trees of *Bambusa vulgaris*. *African journal of biotechnology*, 5: 1245-1248.

Romano A, 1992. Influence of growth regulators on shoot proliferation in *Quercus suber* L. *Annals of botany*, 70: 531-536.

Schroeder KR, Stimart DP, 1999. Adventitious shoot formation on excised hypocotyls of *Antirrhinum majus* L. (Snapdragon) *in vitro*. *Hortscience*, 34:736-739.

Vlahos JC, Dragassaki M, 2000. *In vitro* regeneration of *Ebenus cretica*, L. Proceedings of the IV International Symposium on New Floricultural Crops. *Acta horticulturae*, 541: 305-309.