

Rapid Propagation of *Periploca angustifolia* Labill. by Tissue Culture

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ABSTRACT

Periploca angustifolia Labill. (Asclepiadaceae) is an extremely rare fodder shrub native to Egypt which is being severely affected by habitat loss and overgrazing due to its high palatability to animals. Tissue culture of this species has not been previously reported and may be a method for its conservation and propagation as it is heavily overexploited. An efficient and rapid method for micropropagation of *P. angustifolia* was developed by nodal stem segments collected from mature shrubs in the wild. Nodal explants were established on Murashige and Skoog (MS) basal medium containing 3% sucrose supplemented with different concentrations of 6-benzylaminopurine (BAP) (0.2, 0.5 and 1.0 mg l⁻¹) in combination with β-naphthalene acetic acid (NAA) (0.1 and 0.2 mg l⁻¹). Shoots could be multiplied on MS medium containing 3% sucrose supplemented with BAP (0.5-2.0 mg l⁻¹) and N⁶-(2-isopentenyl) adenine (2iP) (0.5 mg l⁻¹). The maximum number of proliferated shoots was obtained on MS medium containing 3% sucrose supplemented with 2 mg l⁻¹ BAP + 0.5 mg l⁻¹ 2iP. Indole-3-butyric acid (IBA) gave a better rooting response than NAA. Most (70%) shoots rooted on half-strength MS medium containing 3% sucrose and 2.0 mg l⁻¹ IBA to obtain complete plantlets. Eighty percent of the *in vitro* rooted plantlets were successfully hardened in the soil under greenhouse conditions. The use of this method appears to be a promising approach for population reinforcement and for *in vitro* preservation programs of threatened and rare populations.

Keywords: Asclepiadaceae, conservation, *in vitro* culture, micropropagation, nodal explants

Abbreviations: BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2iP, N⁶-(2-isopentenyl) adenine; MS, Murashige and Skoog; NAA, β-naphthalene acetic acid

INTRODUCTION

Periploca angustifolia Labill. from the family Asclepiadaceae is a very rare woody perennial deciduous shrub found in the western Mediterranean coastal region of Egypt. It is known as Wolfbane or African Wolfbane. *Periploca* = "peri" (=around) and "ploke" (=twisted, woven), referring to its twining nature of many species of this Genus (Greek), *angustifolia* = narrow leaves (Latin). This plant is one of the most important species both for its ecological, scientific and conservation importance. Also, it is one of the most palatable plant species to animals. It is a range shrub (energetic value = 0.28 FU Kg⁻¹ DM), with continuous vegetative production when soil humidity allows. It is useful for range rehabilitation. All plant parts, especially the seeds and latex contain various alkaloids and glycosides, many of which are used in medicine and as insecticides. The plant is decreasing rapidly during the past decades due to overgrazing. For this reason it has now been described as a rare plant (Sultana *et al.* 2002; Lanfranco and Lanfranco 2003). Therefore, it is necessary to devise a method for the development of a potentially large-scale multiplication protocol for commercial production of this extremely rare species. It can be introduced successfully in areas with less than 100 mm precipitation, and adapted to different soil types. It can also be planted ideally in the upper reaches of the slopes (Fava *et al.* 1997).

The propagation of *P. angustifolia* in nature is by wind-dispersed seeds but uncontrolled grazing in the wild causes the plant not to complete its life cycle and consequently its seeds are not produced, which result in a decrease in its distribution in nature that ultimately has led to its extreme rarity. Therefore, propagation of *P. angustifolia* both *in situ*

and *ex situ* is a priority for conservation purposes.

In view of the importance and restricted distribution of this species, its propagation and multiplication through tissue culture is urgently required. Plant tissue culture offers advantages over conventional methods for multiplication and large-scale production of woody plants (Thorpe *et al.* 1991). And the *in vitro* propagation of rare and endangered plants can offer considerable benefits for the rapid cultivation of species that are at risk, that have limited reproductive capacity and exist in threatened habitats (Fay 1992). *In vitro* propagation methods offer powerful tools for germplasm maintenance and are also essential components of plant genetic resources management and are becoming increasingly important for conservation of rare and endangered plant species (Iankova *et al.* 2001; Bhatia *et al.* 2002; Almeida *et al.* 2005). Similarly, these techniques facilitate the application of genetic manipulation procedures (Ueno *et al.* 1996; Knapp *et al.* 2001) and long-term storage (Hawkes *et al.* 2000). Although *in vitro* propagation is a potential alternative for the production of *P. angustifolia* for commercial and conservation purposes, until now, no tissue culture studies have been published on this species. The aim of this study was to develop a protocol for *in vitro* propagation of *P. angustifolia* through nodal stem segments culture for effective conservation of this valuable range plant.

MATERIALS AND METHODS

Plant material and sterilization

Nodal segments of *P. angustifolia* were collected from mature plants grown naturally in the western Mediterranean coastal region of Egypt. Nodal segments were chosen for the clonal propagation

of *P. angustifolia* because it was very difficult to obtain newly growing shoots from the plant, as they were continuously grazed by the animals except shoots that animals could not reach (mostly without shoot tips). The explants were washed under running tap water followed by a detergent (Pril) for 5 min. Surface sterilization was done by dipping the explants in 60% (v/v) commercial bleach solution (Clorox) (3% sodium hypochlorite) for 20 min and finally rinsed thoroughly with six changes of sterile distilled water. Explants were cut into single node segments and cultured vertically on sterile nutrient medium.

Culture media and conditions

Explants were cultured on MS basal medium (Duchefa, Haarlem, the Netherlands) (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and gelled with 2.5 g l⁻¹ phytagel (Duchefa, Haarlem, the Netherlands). Different plant growth regulators (PGRs) – cytokinins [6-benzylaminopurine (BAP) and N⁶-(2-isopentenyl) adenine (2iP)] and auxins [β -naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA)] (Sigma Cell Culture, min. 90%, St. Louis, USA), at different concentrations (mg l⁻¹) – either individually or in combination, were added to the medium to optimize hormonal requirements for bud sprouting, multiple shoot induction and rooting, in addition to the control medium without PGRs. The pH of the medium was adjusted to 5.7-5.8 before autoclaving at a pressure of 1.06 Kg cm⁻² and 121°C for 15 min. All cultures were incubated in a culture room at 25 ± 2°C with a 16-h photoperiod under cool white fluorescent tubes (F140t9d/38, Toshiba). Explants were subcultured every 8 weeks. Data were scored after 60 days of multiple shoot induction and rooting.

Culture establishment and shoot multiplication

Various concentrations of BAP (0.2, 0.5, and 1.0 mg l⁻¹) in combination with NAA (0.1 and 0.2 mg l⁻¹) were added to the MS medium for the establishment and bud break. For multiplication of shoots, MS medium was supplemented with 2iP (0.5 mg l⁻¹) singly or in combination with BAP (0.5, 1.0, 1.5 and 2.0 mg l⁻¹).

The percentage of surviving explants, percentage of explants initiating growth, the number of shoots explant⁻¹ and shoot length were measured after 8 weeks of culture. The micropropagation cycle consisted of the regular subculture of nodal segments onto fresh medium.

Effect of seasonal variation on bud break

In order to obtain the best regeneration capacity, the stem node sections were collected several times in four different seasons: April, July, October and January, during 2007-2009. The explants were cultured onto the medium with the best PGR concentration as determined in the establishment experiment.

Rooting and transfer of plantlets to soil

Shoots regenerated from the multiplication stage were excised and transferred to half-strength MS medium supplemented with different concentrations of auxins (NAA and IBA) for *in vitro* root induction. The percentage of rooted shoots, the number and length of rootlets for each rooted microshoot were evaluated after 8 weeks of culture on rooting medium.

Rooted microshoots were removed from the culture medium and the rootlets were washed in sterile distilled water. The plantlets were then transferred to plastic pots containing peatmoss and soil (1: 1) in the greenhouse (28 ± 2°C, 70-80% relative humidity). The potted plants were irrigated and initially covered with plastic bags, which were gradually removed within 4 weeks to complete acclimatization.

Statistical analysis of data

Analysis of variance (ANOVA) and Duncan's multiple range test were performed to analyze the obtained data. At least 10 cultures were raised for each treatment. The differences among means for all treatments were tested for significance at 5% level. Means followed by the same letter are not significantly different at $P \leq 0.05$.

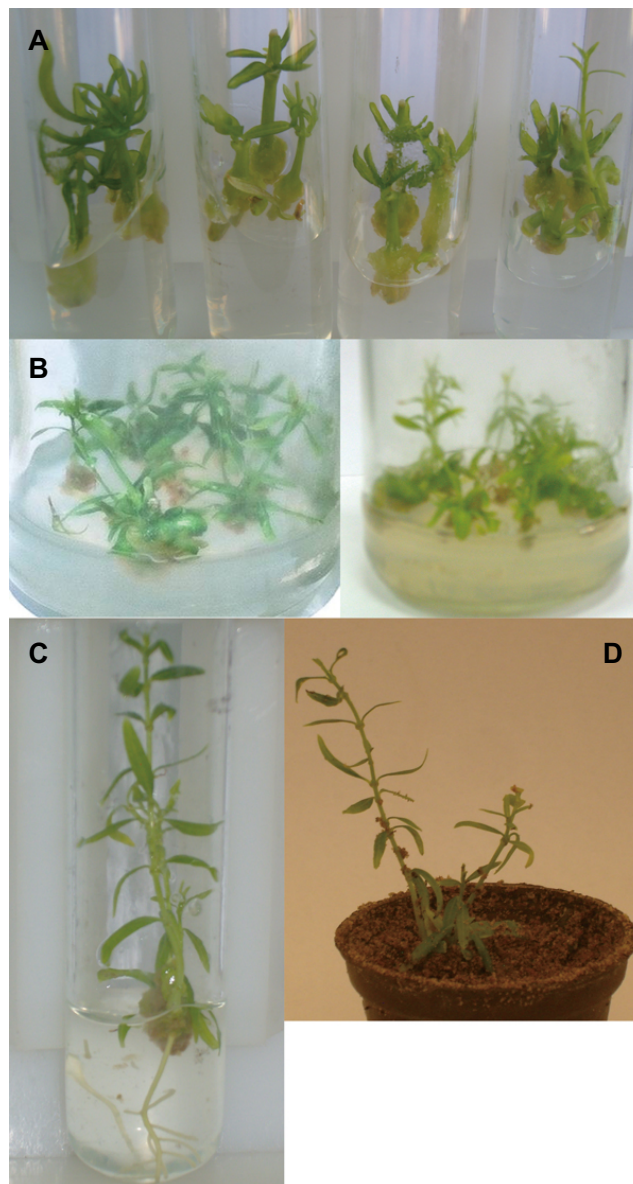


Fig. 1 Micropropagation of *Periploca angustifolia* through nodal explants. (A) Establishment of *P. angustifolia*; (B) Multiple shoot proliferation from axillary nodes after 60 days of culture; (C) *In vitro* rooted plantlet after 60 days of culture; (D) A plantlet acclimatized in soil.

RESULTS AND DISCUSSION

Culture establishment

MS basal medium supplemented with various cytokinin concentrations in addition to NAA showed that axillary shoots could form from stem node sections on all the tested media. However, the percentage of explants growing ranged from 60 to 100% (Table 1, Fig. 1A). Data obtained after 2 months of culture revealed that shoots could be induced on all tested media with 100% growth percentage on MS medium supplemented with 0.2 mg l⁻¹ NAA + 1 mg l⁻¹ BAP, but the survival percentage on this medium was 70% and the average shoot length was 3.9 cm. When compared with the 0.2 mg l⁻¹ NAA + 0.5 mg l⁻¹ BAP medium, both had 90% growth and survival percentages with higher average shoot length (4.2 cm) in the latter. Decreasing concentrations of BAP for each NAA concentration brought about a decrease in the growth percentage. No differences in terms of adventitious shoot formation were observed between 0.2 and 0.5 mg l⁻¹ BAP + 0.1 mg l⁻¹ NAA and 0.2 mg l⁻¹ BAP + 0.2 mg l⁻¹ NAA. By taking into consideration the stem length it was found that among the concentrations tested 0.2 mg l⁻¹ NAA produced a higher average length of shoots than

Table 1 Effect of MS medium and growth regulators (NAA and BAP) on the *in vitro* establishment of *Periploca angustifolia*.

Concentrations (mg l ⁻¹)		Survival (%)	Growth % to survival	Average shoot length (cm)
NAA	BAP			
0.0	0.0	70	60	4.0 c
0.1	0.2	80	70	2.9 d
0.1	0.5	70	70	3.6 cd
0.1	1.0	70	80	3.1 d
0.2	0.2	80	70	5.2 a
0.2	0.5	90	90	4.2 b
0.2	1.0	70	100	3.9 c

0.1 mg l⁻¹ NAA. The highest average length of shoots (5.2 cm) was obtained on medium containing 0.2 mg l⁻¹ NAA supplemented with BAP at 0.2 mg l⁻¹ and it decreased gradually with an increase in BAP concentration. The control medium gave a higher average shoot length (4.0 cm) than 0.1 mg l⁻¹ NAA with different concentrations of BAP.

A synergistic effect of BAP in combination with an auxin has been demonstrated in many medicinal plants of the Asclepiadaceae, *Holostemma annulare* (Sudha *et al.* 1998), *Hemidesmus indicus* (Sreekumar *et al.* 2000), *Holostemma adakodien* (Martin 2002) and *Ceropegia candelabrum* (Beena *et al.* 2003). Also, the stem node section as an explant gave maximum shoot bud sprouting and shoot number from axillary nodes and proved to be better than cotyledonary node and shoot tip explants of *Gymnema sylvestris* (family Asclepiadaceae) (Komalavalli and Rao 2000). *In vitro* propagation of plants belonging to the Asclepiadaceae has also been shown to have optimum overall growth in MS medium (Chi Won and John 1985; Patnaik and Debata 1996; Komalavalli and Rao 1997). In addition, Komalavalli and Rao (2000) found that MS salts for shoot sprouting and proliferation showed a high salt requirement for the growth of *G. sylvestris*. Thus, the degree of growth and differentiation varied considerably with the medium constitution (Shekhawat *et al.* 1993; Das *et al.* 1996).

Effect of seasonal variation on survival and bud break

The effect of seasonal variation on survival and growth percentage and average shoot length was investigated after determining the optimum cytokinin and auxin levels for shoot sprouting in order to resolve the best season for culture establishment. Data in **Table 2** reveals that spring is the most suitable season in terms of survival percentage (60%), growth percentage (100%) and average shoot length (4.5 cm). These results are in agreement with those obtained by Hegazi (2000) who found that the explants of *Capparis cartilaginea* and *Nitraria retusa* collected after the growing season exhibited the highest percentage of contamination compared to explants collected from new shoots growing in spring. Also, the time of the year in which the explant is taken affects the results of a micropropagation program because of the variation in the physiology of the plant during different seasons. This may be due to changes in temperature, day length, light intensity and water availability throughout a year which affect the levels of carbohydrates, proteins and growth substances in plants. Hegazi (2000) reported that the newly growing shoots of *C. cartilaginea* and *N. retusa*, which were taken during the active phase of growth (spring) contained markedly high levels of endogenous phytohormones compared to the other seasons.

Shoot multiplication

In order to increase the number of shoots explant⁻¹, shoots were exposed to 6 treatments of MS basal medium supplemented with BAP at 0.0, 0.5, 1.0, 1.5 and 2.0 mg l⁻¹ combined with 2iP (0.5 mg l⁻¹), in addition to the control. Combination of the two cytokinins was more ideal compared to 2iP singly, producing fewer shoots (**Table 3, Fig.**

Table 2 Effect of seasonal variations on the survival and growth of *Periploca angustifolia in vitro*.

Season	Survival %	Growth % to survival	Average shoot length (cm)
Spring	60	100	4.5 a
Summer	50	90	3.7 b
Autumn	50	80	3.2 c
Winter	40	60	3.0 c

Table 3 Effect of MS medium and growth regulators (BAP and 2iP) on the *in vitro* shoot proliferation of *Periploca angustifolia*.

Concentrations (mg l ⁻¹)		Average no. of shoots/explant	Average shoot length (cm)
BAP	2iP		
0.0	0.0	2.00 e	4.6 a
0.0	0.5	2.50 e	4.0 b
0.5	0.5	5.40 d	3.7 b
1.0	0.5	7.00 c	3.1 bc
1.5	0.5	9.80 b	2.5 c
2.0	0.5	12.3 a	2.2 c

Table 4 Effect of ½ MS medium and growth regulators (IBA and NAA) on the *in vitro* rooting of *Periploca angustifolia*.

Concentrations (mg l ⁻¹)		% of explants forming rootlets	Average no. of rootlets/explant	Average rootlet length (cm)
IBA	NAA			
0.0	0.0	0.0	0.0	0.0
1.0	0.0	60	8.0 b	5.4 b
2.0	0.0	70	12 a	8.3 a
0.0	1.0	40	6.0 c	4.9 c
0.0	2.0	50	8.0 b	5.5 b

1B).

Shoot multiplication rates were significantly affected by the concentration of BAP as up to 12.3 shoots explant⁻¹ were recorded using 2 mg l⁻¹ BAP + 0.5 mg l⁻¹ 2iP. Longest shoots were obtained in the control treatment, whereas the application of BAP and 2iP significantly decreased shoot elongation. Thus, direct plant regeneration from stem explants could be an alternative to obtain *in vitro* plantlets of *P. angustifolia* as plant regeneration from stem explants of woody species were morphologically normal (Palacios *et al.* 2002).

Rooting and ex vitro acclimatization

Stem microcuttings excised from multiple shoot cultures rooted on half-strength MS medium supplemented with an auxin within 8 weeks. From data presented in **Table 4** and **Fig. 1C** it is clear that IBA was significantly the more efficient auxin type for adventitious rooting than NAA. The highest percentage of explants producing rootlets (70%) was observed on medium containing 2 mg l⁻¹ IBA and rootlets reached 12 explant⁻¹ and elongated to 8.3 cm in length. Medium supplemented with 1 mg l⁻¹ IBA gave the second best response by exhibiting 60% of rootlets formation and 5.4 cm root length. Half-strength MS medium free of PGRs decreased rooting percentage. This result is in harmony with that obtained by Đurković (2008). Also, Komalavalli and Rao (1997, 2000) noticed that of 3 auxins, indole-3-acetic acid (IAA), IBA and NAA, tested to induce rooting of *Gymnema* sp., IBA (3 mg l⁻¹) was most effective for root induction and survival in the field. The use of half-strength MS medium for root induction of *P. angustifolia* was supported by Beena *et al.* (2003) who reported that half-strength MS PGR-free medium induced more roots compared to full-strength MS in *Ceropegia candelabrum* (Asclepiadaceae).

In the root meristem, auxin is implicated in regulating the pattern of cell division and differentiation (Friml 2003). According to Puente and Martin (1997), if the shoots are competent to root, rooting rate could be increased easily. It has been reported that shoot characteristics such as size and shoot culture origin fail to attain a stabilized growth phase

or apparent rejuvenation can also lead to a variable rooting response (Marks and Simpson 2000).

Rooted plantlets were successfully acclimatized in 4 weeks in simple plastic pots in a peat moss and soil mixture (1: 1). They did not show any detectable morphological variation (Fig. 1D).

CONCLUSION

The outlined procedure offers a potential system for conservation and mass propagation of *P. angustifolia* from nodal explants. MS medium containing 0.2 mg l⁻¹ NAA + 0.5 mg l⁻¹ BAP is best for establishment and 0.5 mg l⁻¹ 2iP + 2 mg l⁻¹ BAP for shoot proliferation. Half-strength MS basal medium supplemented with 2 mg l⁻¹ IBA is optimum for root induction. The *in vitro* propagation of *P. angustifolia* is not very difficult and could be applied to conserve this important rare species.

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