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Micropropagation of *Pelargonium sidoides*

Francis B. Lewu, Don S. Grierson and Anthony J. Afolayan*
Department of Botany, University of Fort Hare, Alice, 5700, South Africa

Abstract

Pelargonium sidoides is a medicinal plant harvested for local and export trades in South Africa. In order to contribute to the conservation and propagation initiatives of the herb, a study on the micropropagation of the plant was conducted. Our findings revealed that callus induction was achieved by using different concentrations of IBA and 2,4-D. Optimal response was obtained at 3 mg l^{-1} IBA under continuous dark condition. The study also showed that shoot differentiation is achievable from stem derived callus after one week in hormone free media under continuous light. The percentage of callus formation ranged from 0 to 88.9% depending on the type of auxin used, the concentration and photogenic conditions. Transferred plantlets by simple acclimatization procedure survived and produced healthy plants. The significance of these on the propagation and conservation of *P. sidoides* is highlighted in the discussion.

Keywords: Callus induction; Eastern Cape; *ex situ* conservation; growth regulators; medicinal plants; micropropagation; *Pelargonium sidoides;* South Africa.

Introduction

Herbal medicine is widely used in South Africa and most of the plants used for this purpose are harvested from the wild populations. Apart from local uses of medicinal plants, there is a current upsurge in their harvesting by the rural communities mainly for local and international trades (Cunningham, 1988; Lange, 1997). This practice has negative affect on plant demography especially in unprotected lands (Ake-Assi, 1988; Kokwaro, 1991; Cunningham and Mbenkum, 1993; O'Brien and Kinnaird, 1996; Gu, 1998; McKean, 2003; Ghimire *et al.,* 2005; Tang *et al.,* 2005). Among such plant species facing the danger of over-harvesting is *Pelargonium sidoides* DC.

Pelargonium sidoides is used by the people of Eastern Cape for the treatment of various diseases in human and livestock (Batten and Bokelmann, 1966). The use of this plant for the treatment of various diseases by the people of Eastern Cape has been validated through microbial bioassay (Lewu *et al.,* 2006a). In Germany, Schwabe Pharmaceutical produces *Umckaloabo* from the roots of *P. sidoides* imported from South Africa. The product is said to be effective against bronchitis in children (Van Wyk *et al.,* 1997; Matthys *et al.,* 2003; Van Wyk and Gericke, 2003; Donald and Brown, 2004). Due to the high demand for the species, the rate of harvesting has exceeded the rate of natural regeneration (Lewu *et al.,* 2007a). Our preliminary study indicated that the viability of seeds collected from the natural population is very low coupled with low seed germination. In continuation of our effort to promote the conservation and propagation of this plant both *in situ* and *ex situ* (Lewu *et al.,* 2006b, c), we report our findings in this paper, the initiation of tissue culture protocol for the micropropagation of the species.

Materials and Methods

Plant materials

The experiments were carried out in the phytomedicine laboratory of the Department of Botany, University of Fort Hare, South Africa. Explants were raised from leaf, seed and stem sources. For stem and leaf cultures, plants raised in the greenhouse were used as the source of primary explants. While, balked seeds from the harvest of early study (Lewu *et al.,* 2007b) were used as source of seed explants. Seeds were surface sterilized with 70% ethanol for two minutes, and 0.1% mercuric chloride for 15 minutes. The sterilized seeds were rinsed several times with double distilled sterile water and soaked for 24 h. The swollen mature embryos were removed from seeds with a scalpel and were then plated cut-side down on induction media. The same sterilization procedure was conducted on the leaf and stem cultures. However, explants were treated in 0.1% mercuric chloride for five minutes and rinsed in several exchanges of double distilled sterile water before culturing.

Callus induction

The callus induction medium contained Murashige and Skoog's (1962) basal salts, supplemented with 1.0 - 4.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) or indole-3-butyric acid (IBA), Na_2EDTA (7.4g.l^{-1}), myo-inositol (20 g l^{-1}), thiamine-HCl (0.1 g l^{-1}), 2.0 mg l^{-1} glycine, 690 mg l^{-1} proline, sucrose (30 g l^{-1}) and was solidified with 5 g l^{-1} Difco bacto-agar. The pH was adjusted to 5.8 and the media were sterilized by autoclaving at 121°C for 20 min. The embryos (from seeds), leaf and stem explants were incubated for callus induction in the media at 25 ±3°C under continuous illumination with a photosynthetic photon flux density of 184.8 (±5) μmol m^{-2} s^{-1} provided by cool-white

*Corresponding author: Aafolayan@ufh.ac.za

fluorescent lamps. The same experiment was duplicated under continuous dark condition in three replicates. Twenty four mature embryos were inoculated per treatment making a total of 48 samples for both light and dark experiments. The same was repeated for leaf and stem explants. After three weeks of culture in induction medium, the percentage of mature embryos, leaf and stem explants producing primary calli were determined, and the calli were then cut into smaller sizes and transferred to the same medium for another two weeks.

Callus subculture, shoot differentiation and rooting

The basal composition of the subculture medium was the same as that of the induction medium except for the removal of plant growth regulators and the reduction of agar to 4 g l^{-1}. Each callus was cut into smaller pieces (approximately 0.5g fresh weight) during transfer and subcultured three times. The cultures were transferred onto fresh subculture medium every two weeks and were maintained at 25 ±3°C under continuous illumination. After six weeks, the percentages of calli forming shoots were recorded. Rooting percentage, number and length of roots were recorded after six weeks on the hormone free media. At about 4 cm height and with four visible leaves, plantlets with healthy-looking roots were removed from culture, rinsed in water (to remove media) and transplanted into a mixture of equal parts (v/v) of sterilized soil and vermiculite. They were watered with half-strength MS salts solution and acclimatized under humid conditions in plastic pots.

Data analysis

The callus induction experiment was analyzed in a factorial pattern with growth regulators and light being the main factors. Two auxins (2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-butyric acid (IBA) at four levels each were tested under continuous light and darkness conditions. The first data were analyzed using a proc GLM model of the SAS package in a factorial arrangement. Duncan Multiple Range test (P < 0.01) was used for multiple mean comparisons of the interactions between the different levels of auxins and photogenic conditions. In the second experiment, only callus derived from stem source differentiated to shoot after two subcultures in auxin-free media in the continuous light condition. The analysis of the data was performed using proc GLM analysis and means were separated by LSD (SAS, 1999).

RESULTS

Influence of auxins and light on callus formation

Table (1) shows the effect of auxins on the formation of callus from seeds and stems of *Pelargonium sidoides*.

Table (1): Influence of auxins on the formation of callus from the seeds and stems of *Pelargonium sidoides* after three weeks in culture.

Auxins (mg l^{-1})	Mean value of callus from seed and stem (%)
indole-3-butyric acid (IBA)	
1	*18.1[b]
2	4.2[d]
3	63.9[a]
4	0[e]
2,4-dichlorophenoxyacetic acid (2,4-D**)**	
1	11.1[c]
2	13.9[c]
3	0[e]
4	0[e]

Values followed by the same letter in the column are not significantly different at *P<0.01*. *The result shown above is a statistical mean value (n=3) of the percentage yield of *P. sidoides* as influenced by auxins at different concentrations

The percentage of callus formation ranged between 0 to 63.9% with 3 mgl^{-1} of IBA being the optimum concentration in the study. Table 2 shows the morphogenic response of seed and stem explants of *P. sidoides* to the two auxins under two photogenic conditions in a factorial arrangement. The percentage of callus formation ranged between 0 to 88.9% depending on the type of auxin, the concentration, source of explant used, and photogenic conditions. The interesting part of the results is the interaction between the different levels of auxins and the two levels of light conditions considered in the study. Apart from cases where no callus were formed, statistical analysis showed significant (P < 0.01) interactions between all levels of auxins and the two light conditions tested in the study. Callogenesis was generally influenced by auxins and photogenic conditions. However, IBA at 3 mg l^{-1} and under continuous dark condition demonstrated the optimum conditions for callus formation especially in seed explants (88.9%) and the best yield obtained from 2,4-D (19.5%) was under continuous light condition. Seed callus had the highest weight of 1.6 g and about 2 cm radius after 21 days in initiation media (Fig. 1A).

Shoot differentiation, root formation and acclimatization

Two initial subcultures of callus from the two explants (seed and leaf) sources in their respective auxins did not produce any shoot. After two repeated cultures in auxin free media, the callus formed from seed and leaf sources did not differentiate to shoot either. However, stem derived calli produced shoots (average of two shoots per callus) in auxin free full MS medium in the first subculture (Table 3 and Fig. 1B). In this study, the rate of stem formation from callus was very low with an optimum of 27.8% in 3 mg l^{-1} IBA derived callus followed by 2 mg l^{-1} 2,4-D. Explants derived from 3 mg l^{-1} IBA demonstrated significant (P < 0.01) yield advantage over others, thereby producing more healthy plantlets. Rooting was

Table (2): Effects of the factorial interaction of auxins and light on the formation of callus from the seeds and stems of *Pelargonium sidoides* after three weeks in culture.

Auxins (mg l^{-1})	Light condition[a]	Callus formed from seed explant (%)	Callus formed from stem explant (%)
indole-3-butyric acid (IBA)			
1	+	13.9*	0
2	+	8.3	0
3	+	38.9	44.5
4	+	0	0
1	–	22.2	8.3
2	–	0	0
3	–	88.9	44.5
4	–	0	0
LSD		*6.34*	*5.16*
2,4-dichlorophenoxyacetic acid (2,4-D)			
1	+	8.3	8.3
2	+	19.5	8.3
3	+	0	0
4	+	0	0
1	–	13.9	0
2	–	8.3	11.1
3	–	0	0
4	–	0	0
LSD		*3.16*	*2.58*

[a] + indicates continuous light and – indicates continuous darkness. *There was significant (*P<0.01*) interaction between the two levels of light and both auxin levels tested except for values with zero. All values are presented as means of three replicates.

Table (3): Initiation of shoots and roots from stem derived callus after three weeks in culture.

Auxins (mg l^{-1})	Callus differentiating to stem (%)	Average length of roots (cm)	Average number of roots
indole-3-butyric acid (IBA)			
1	8.3[b]		
2	0[c]	6.6[b]	1[b]
3	27.8[a]		
4	0[c]	7.4[a]	1[b]
2,4-dichlorophenoxyacetic acid (2,4-D)			
1	0[c]		
2	11.1[b]		
3	0[c]	3.2[c]	2.7[a]
4	0[c]		

Values followed by the same letter in the column are not significantly different at *P<0.01.* All values are presented as means of three replicates.

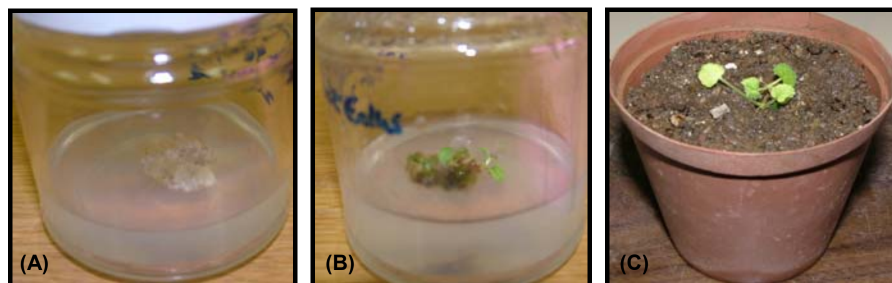

Figure (1): (A) Callus formed from the seed of *Pelargonium sidoides*, **(B)** Shoot formed from tem derived callus two weeks after subculture, and **(C)** Transplanted explant of *Pelargonium sidoides* after six weeks in media.

interestingly achieved in the hormone free media with an average of 1 to 2.7 roots per plant. All the plantlets derived from IBA treated explants produced a single, long and thin root; while plantlets derived from 2,4-D produced between two and three but shorter root systems (Table 3). Plantlets transferred by simple acclimitation procedure involving the use of equal volume (v/v) of sterilized soil and vermiculite produced healthy plants (Fig. 1C).

DISCUSSION

IBA generally showed good potential for callus initiation in *P. sidoides*. With the result obtained from this experiment, it appears that callus formation in this

plant could be impaired from any concentration above 3 mg l^{-1} as the explants did not produce any callus above this concentration in both auxins used. Unlike other reports on many species (Chang *et al*., 2000; Cucco and Rossi, 2000; Wang *et al*., 2003; Feeney and Punja, 2003), 2,4-D generally showed limited influence on callus formation in *P. sidoides* with the highest percentage being 13.9% at 2 mgl^{-1} (Table 2). This concentration is however, consistent with the general range of concentration (1.0-3.0 mgl^{-1}) reported in the literature. The study also showed that shoot differentiation is achievable from stem derived callus after one week in auxin free media under continuous light condition. Conventionally, this species perennates itself in the wild through the under ground root systems (Lewu *et al*., 2007a). The ability to produce roots without rooting hormone may be connected to this innate quality.

Micropropagation techniques have been one of the recently used tools for the propagation and conservation of threatened and endangered plant species (Castillo and Jordan, 1997; Saxena *et al.,* 1997; Murch *et al*., 2000). With increasing demand for herbal based medicines in the local and international market (Cunningham, 1988; Lange, 1997), micropropagation technique has become a necessary tool for the conservation and multiplication of several plant species. Our findings revealed that Callus induction was achieved by using different concentrations of IBA and 2,4-D. Optimal response was obtained by the use of IBA at 3 mg l^{-1} under continuous dark conditions. This study is more particularly important for medicinal plant species which may not recover for a long time following uncontrolled harvesting of their medicinally important underground bulbs and roots (Cunningham, 1991; Rock *et al.,* 2004; Lewu *et al*., 2007a). The development of micropropagation technique for this herb will further promote *ex situ* conservation initiative through propagation programme for the species, thereby removing pressure from wild population.

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