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In Vitro Shoot Organogenesis from *Pelargonium* × *Citrosum* Vanleenii Leaf and Petiole Explants

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ABSTRACT

Mosquito plant (*Pelargonium* × *citrosum* Vanleenii) is a genetically engineered hybrid possessing the characteristics of geranium coupled with a sweet lemony citronella scent. As such, this hybrid has become much more popular as an ornamental plant with the added benefit of mosquito-repelling capability. We report a novel protocol for shoot organogenesis from young leaf and petiole explants sourced from both pot grown and *in vitro* plants. Our study shows that 6-benzyladenine, thidiazuron and kinetin are effective in inducing adventitious shoot production, unlike 2-4-dichlorophenoxyacetic acid and α-naphthaleneacetic acid. This suggests that these cytokinins play a key role in inducing adventitious shoots from young *P.* × *citrosum* leaf and petiole explants. This study offers a novel and efficient mass shoot propagation and plant regeneration protocol for mosquito plant.

Keywords: cytokinin, mosquito plant, plant growth regulator, shoot organogenesis
Abbreviations: 2,4-D, 2-4-dichlorophenoxyacetic acid; **BA**, 6-benzyladenine; **IBA**, 3-indolebutyric acid; KIN, Kinetin **NAA**, α-naphthaleneacetic; acid; **TDZ**, thidiazuron

INTRODUCTION

The worldwide threat of mosquito-transmitted diseases, with their associated mortality, especially in tropical and subtropical developing countries, highlights the need for environmentally responsible and efficient insect repellents. Natural insect repellent products are relative safe around humans (Quarles 1996; Zaridah *et al*. 2006). The only compound besides DEET (*N*,*N*-diethyl-3-methylbenzamide) registered for human use as an insect repellent is citronella derived from the citronella plant (Lindsay *et al*. 1996; Brown and Hebert 1997; Mark and Fradin 1998). Mosquito plant (*Pelargonium* × *citrosum* Vanleenii) is a genetically engineered geranium hybrid by cell fusion that incorporated the trait of citronella grass and a scented geranium (Raver 1991; Tucker and Maciarello 2003). The resulting cultivar has the appearance of geranium combined with a sweet lemony citronella scent (Matsuda *et al*. 1996; Murugan and Jeyabalan 1999; Jeyabalan *et al*. 2003). This combination of attractive foliage, sweet lemony citronella scent and mosquito repelling activity makes the mosquito plant a very popular ornamental plant all over the world. Mosquito plants can flower but do not produce seed, so tissue culture can be used to enhance clonal propagation. In recent years mosquito plant has been mass propagated for commercial purposes with an estimated one million plants supplied to Asia every year. So it is much necessary to establish an efficient mass propagation and plant regeneration system in mosquito plant. Regeneration of *Pelargonium* × *hederaefolium* from petiole explants and *in vitro* multiplication of *Pelargonium* cultivars have been reported (Wojtania and Gabryszewska 2001, 2004); mass propagation and regeneration through apical or axillary bud culture in *Pelargonium* × *citrosum* have also been studied (Jiang and Liu 2004; Wei *et al*. 2005). Somatic embryogenesis has been achieved with hybrid geranium (*Pelargonium* × *hederaefolium*) (Gill *et al*. 1992; Hutchinson and Saxena 1996; Hutchinson *et al*. 1996; Borja and Alonso 2006) and *Pelargonium* × *hortorum* Bailey (Qureshi and Saxena 1992; Visser *et al*. 1992). However, shoot organogenesis from cultured leaf or petiole explants has not been reported with *P.* × *citrosum*. As leaf and petiole material is relatively abundant, we considered the possibility that this type of material would be very convenient to source and use provided a suitable method for organogenesis could be devised. In this study, leaf and petiole explants of *P.* × *citrosum* sourced from both potted and *in vitro* plants were used to investigate specific effects of plant growth regulators on *de novo* shoot induction with the aim of providing an efficient plant regeneration and mass propagation protocol for this important horticultural species.

MATERIALS AND METHODS

Plant material and induction of adventitious shoots

Six-month-old potted plants (15 cm high) of *Pelargonium* × *citrosum* were sourced from South China Botanical Garden. Leaf segments (0.3 cm^2), young apical buds and petioles (0.5 cm long) were excised and consecutively surface sterilized by rinsing in 70% (v/v) alcohol for 10 s, immersing in 0.1% (w/v) mercuric chloride for 8 min and finally rinsing 3 times with sterile distilled water. Explants were cultured for 5 weeks in darkness on MS (Murashige and Skoog 1962) basal medium containing 1.0 mg l^{-1} 6-benzylaminopurine (BAP) + 0.2 mg l^{-1} naphthalene acetic acid (NAA). The medium was adjusted to pH 5.8 and solidified with 0.6% (w/v) agar. The culture jars were 10 cm high and 5 cm in diameter and was dispensed usually 20 ml media. After 20 days calli of 5-8 mm in size with adventitious shoots formed were transferred to the same medium (as above). The cultures were grown in a cultivation chamber at 26 ± 1°C with 14 h of cool white fluorescent light at photon flux density 50 μmol m^{-2} s^{-1} daily.

Received: 13 July, 2007. Accepted: 9 August, 2007.

Short Communication

Induction of adventitious shoots *in vitro*

Following the establishment of tissue culture, young leaf and petioles explants from *in vitro* grown plants were used to study the induction of callus and adventitious shoots. Media for this experiment consisted of MS basal mineral salts as described above, supplemented with 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} thidiazuron (TDZ), 1.0 mg l^{-1} BAP, 1.0 mg l^{-1} 6-furfurylaminopurine (KIN), 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, respectively. Induction of adventitious shoot formation was studied. Fifty explants were used for the experiments with each variant of the media and the results were averaged for two replications of each variant. Post hoc LSD test at $p \leq 0.05$ (SPSS 12.0 for Windows) was used for comparison of means.

Mass propagation and plant regeneration

Callus clumps with adventitious shoots were transferred to medium containing 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA for mass propagation and shoots were subcultured in the same medium every two months. The multiplication index was calculated based on shoot proliferation rates averaged over five subculture periods.

Induction of adventitious roots

Two hundred shoots (in 2-3 cm high with 2-3 leaves) were transferred for rooting on half-strength MS media containing 0.2 mg l^{-1} indole-3-butyric acid (IBA) and also no plant growth regulator, respectively. The tests were repeated twice again in one week. Culture conditions were the same as above.

Acclimatization

One month following incubation on root induction medium, a total of 200 plantlets from 0.2 mg l^{-1} IBA containing medium were removed from jars and after agar was washed from the roots, they were transplanted to sand beds in the greenhouse in March. The test was repeated once again in one week. The survive rate was investigated after two weeks of transplanting. The temperature in the greenhouse was between 20-25°C. Spraying in the soil with 1/3 MS mineral salts solution (macro and micro elements) was applied once a week. After two weeks of acclimatization plants were transferred to pots (10 cm high, 12 cm in diameter) containing a mixture of sand and peat (1:1 v/v) or sand and humus soil (1:1 v/v), respectively. The survive rate was investigated after one month of transferring to pots.

RESULTS AND DISCUSSION

Induction of shoot organogenesis from young leaf segments and petioles from potted plants

Apical buds cultured on induction medium (1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA), proliferated to form multiple-shoot clusters. However, callus tissue was induced from cut surfaces on leaf segments and petioles within two weeks (**Fig. 1A**). Prolonging culture time to 4-5 weeks resulted in shoot organogenesis with highly visible apical bud structures on the callus surface (**Fig. 1B**). It was also observed that some adventitious shoots developed directly on the leaf veins without callus formation.

Shoot organogenesis from young leaf segments and petioles *in vitro*

Leaf segments and petiole explants cultured in darkness on induction medium containing 1.0 mg l^{-1} 2,4-D exhibited yellow callus formation on the cut surfaces within two weeks. Following prolonged culture to 4-5 weeks, the callus proliferated slowly and generally turned brown, with no adventitious shoots observed.

On induction medium containing 1.0 mg l^{-1} NAA, leaf segments or petioles became swollen and very few calli were induced on the cut surfaces within one week, however some calli developed white hairy roots. No shoot was observed.

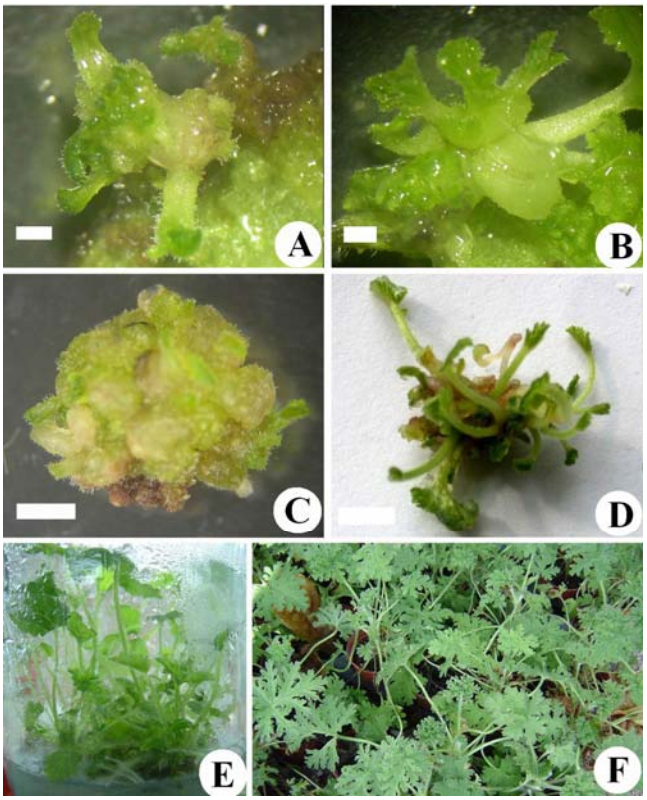

Fig. 1 Adventitious shoot formation from young leaf and petiole explants of *P.* × *citrosum*. (**A, B**) Adventitious shoots were induced on the petiole explants; (**C**) Callus clump with adventitious shoot formation after four weeks of culture on induction medium containing 1.0 mg l^{-1} BAP; (**D**) Adventitious shoot formation after six weeks in culture; (**E**) *In vitro* shoot mass propagation and root formation; (**F**) *In vitro* adventitious shoot-issued plantlets transplanted in pots. Bar length = 2 mm.

The media containing either 1.0 mg l^{-1} KIN, TDZ or BAP alone induced yellow-white callus within two weeks, then some yellow-white adventitious shoots developed on the callus during the following week (**Fig. 1C**). With the culture time prolonged to 4-6 weeks, all adventitious shoots developed into multiple shoots (**Fig. 1D**). BAP and TDZ induced significantly more adventitious shoots than KIN (**Table 1**).

Combining BAP and NAA in the induction media also induced shoot organogenesis resulting in adventitious shoot formation from leaf segments and petioles. However, different concentrations of BAP or NAA in the media appear to induce different callus types. Medium supplemented with 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA induced friable, waterlogged callus, from which vitrified adventitious shoots originated. However, medium supplemented with 0.2 mg l^{-1} BAP + 1.0 mg l^{-1} NAA induced compact callus with normal adventitious shoot formation (**Table 1**).

Mass propagation and plant regeneration

Adventitious shoots cultured on propagation medium produced a mean multiplication rate of 6.8 ± 0.5 per eight weeks incubation period indicating very high propagation efficiency.

After one week on root induction medium the shoots began forming of roots, and after four weeks all shoots developed roots (**Fig. 1E**). However, as the medium contained no plant growth regulator, some roots occurred after cultured for three weeks. Four weeks later, all the shoots developed roots on the IBA medium. However, only 46 ± 2% of the shoots could develop root formation on the medium free of IBA (**Table 2**).

Table 1 Effect of plant growth regulators on adventitious shoot induction in *in vitro*-derived young leaves and petioles of mosquito plant.

Plant growth regulators (mg l^{-1})	Observation Results within 6 weeks	Number of adventitious shoot per explant
0	No callus	0 a
2,4-D 1.0	Callus	0 a
NAA 1.0	Hairy root callus	0 a
TDZ 1.0	Callus, adventitious shoots	12.6 d
BAP 1.0	Callus, adventitious shoots	11.8 d
KIN 1.0	Callus, adventitious shoots	6.7 c
BAP 1.0 + NAA 1.0	Friable and waterlogged callus, vitrification of adventitious shoots	4.6 b
BAP 1.0 + NAA 0.2	Callus, adventitious shoot	13.5 d
BAP 0.2 + NAA 1.0	Rooting callus, adventitious shoots	4.3 b

The same letters indicate no significant differences between means (LSD test, $p \leq 0.05$)

Table 2 Root formation and plantlet transplant test of mosquito plant

Root formation and plantlet transplanting	Observation in different culture media	
	Control	IBA-treated
Time for root formation (weeks)	3	1
Root fomation in 4 weeks (%)	46 b	100 a
Survive rate in the sand	No test	88 ± 2%
Survive rate transplanting to pots of sand and peat (1:1)	No test	99 ± 1%
Survive rate transplanting to pots of sand and humus (1:1)	No test	98 ± 1%

The same letters in the same column indicate no significant differences between means (LSD test, $p \leq 0.05$)

A high percentage 88 ± 2% of rooted plants from IBA contained medium survived after they were transplanted to greenhouse conditions after two weeks. As they were transferred to the pots with sand and peat (1:1) or sand and humus (1:1), above 97% of plants can survive to grow normally, no obvious aberrations were observed (**Fig. 1F, Table 2**).

Apparently, most of the induction media tested could induce shoot organogenesis and adventitious shoot formation from leaf and petiole explants of the mosquito plant. Media containing TDZ, BAP alone or BAP as a dominant plant growth regulator could induce more adventitious shoots than other PGR combinations tested. The results are in agreement with previous reports on other species such as watermelon, almond and Mickey Mouse plant (Compton and Gray 1993; Ainsley *et al*. 2000; Ma and Wu 2006), they could be induced adventitious shoot formation from leaf or cotyledon explants with cytokinin TDZ and BAP. Our results from this study indicate that the cytokinin TDZ and BAP can also play a major role in shoot organogenesis from leaf and petiole tissues in the mosquito plant. However, TDZ could also induce somatic embryogenesis from leaf of geranium (*Pelargonium* ×*hortorum* Bailey) (Qureshi and Saxena 1992; Visser *et al*. 1992), this showed some differences on induction system in different genus of *Pelargonium.* We contend that the present study demonstrates the increased opportunities for greater efficiency in plant regeneration and mass shoot proliferation in *P.* × *citrosum* that would possibly enhance commercial production of this important horticultural plant.

ACKNOWLEDGEMENTS

Support by the Program of Digital Botanical Garden and Tropical and Subtropical Plant Germplasm Construction in Guangdong Province (2005B60301001) is gratefully acknowledged.

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