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Establishment and *In Vitro* Propagation of a Putative Variant of Periwinkle

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المزارع النسيجية وإكثار الطفرة المحتملة لنبات البفته (الونكا)

الملخص: تم إنتاج النمو الخضري للطفرة مختبرياً من نبات البفته (الونكا) بأستخدام قمم النموات الخضرية و البراعم الأبطية كأجزاء نباتية مبد ائية. حفز إضافة منظمات النمو النباتية لوسط التأسيس و تفتح البراعم وإستطالة النموات الخضرية الجديدة. بينت النتائج أن أعلى تضاعف (١٥,١ نمو خضري/جزء نباتي) وأطول نمو خضري (٧,٠ سم) حدث في وسط موراشيجي وسكوج المحتوى على ١,٠ ملجم من كل من هرمون البنزيل أدينين و نفثالين حمض الخليك. كونت معظم النموات الخضرية النسيجية جذوراً (١٠٠%) عندما زرعت على نصف تركيز أملاح بيئة موراشيجي وسكوج المضاف إليها ٠,٥ ملجم نفثالين حمض الخليك أو أندول حمض البيوتريك. تم أقلمة النباتات المجذرة بالأنابيب بنجاح حيث بلغت نسبة النجاح حوالي ٩٥% عند نقلها للتربة.

ABSTRACT: Shoot multiplication of a putative variant of *Catharanthus roseus* (L.) G. Don, was achieved in vitro using shoot tips and nodal segments as explants. The addition of growth regulators to establishment medium stimulated bud breaking and shoot elongation. The maximum shoot multiplication (15.1 shoots/microshoot) and the longest shoots (7.0 cm) occurred on Murashige and Skoog medium (MS) containing 1.0 mg L^{-1} of N6-Benzyladenine (BA) and α-Naphthalene acetic acid (NAA). All microshoots formed roots and normal root morphology occurred on half strength MS salt supplied with 0.5 mg L^{-1} NAA or Indole-3-Butyric acid (IBA). Rooted microshoots (95%) were successfully transferred to soil.

The periwinkle, *Catharanthus roseus* (L.) G. Don, is a perennial plant and a member of *Apocynaceae* family. The species can grow in many parts of the world and is mainly cultivated as an ornamental plant (Stearn, 1975; Hirata *et al.*, 1994). It is an important medicinal plant with many uses (Morton, 1976). Thus, it has been extensively investigated for its alkaloid contents and their pharmacological activity. However, *in vitro* propagation has attracted little attention (Cordell, 1980; Stapfer and Heuser, 1985). Cell and tissue culture have been exploited to study synthesis and alkaloid production of periwinkle and others (DiCosmo, 1990; Kutney, 1990).

A variant plant was observed in the garden of the College of Agriculture and Veterinary Medicine, King Saud University. The growth habit of this variant is completely different from the normal plant. Seeds of normal *C. roseus* L. were treated with gamma rays, but full information about the irradiation treatment can not be obtained. The variant is characterized by its rosette and compact shape. It has many branches, each branch also ends with many branches forming a head with many sub-branches and leaves. The plant does not flower, but the vegetatively growing plant is a source of attractive landscaping (Figure 1). The plant can only be propagated vegetatively. Thus, a study was initiated to propagate this putative variant through tissue culture techniques and then to use the tissue-cultured plants for further investigations and studies of this variant.

Materials and Methods

ESTABLISHMENT OF ASEPTIC CULTURES: Newly growing branches were collected from the variant plant. Shoot tips and nodal segments were excised and washed thoroughly with sterile distilled water containing a few drops of Dermosept solution (4% Chlorhexidine Gluconate, SPIMACO, Saudi Arabia). They were then surface-sterilized by dipping in 70% ethanol for few seconds followed by successive agitation in 10% commercial bleach solution (5.25% sodium hypoclorite) for 10 min and 0.02% $HgCl_2$ for 5 min. Few drops of Tween 20 were added to the sterlization solutions. After washing four times with sterile distilled water in a laminar flow hood, explants were cultured on Murashige and Skoog (MS) (1962) medium containing 3.0% sucrose without growth regulators or supplemented with 1.0 mg L^{-1} BA, 0.1 mg L^{-1} Gibberellic acid (GA_3), and 0.1 mg L^{-1} IBA. Twenty-

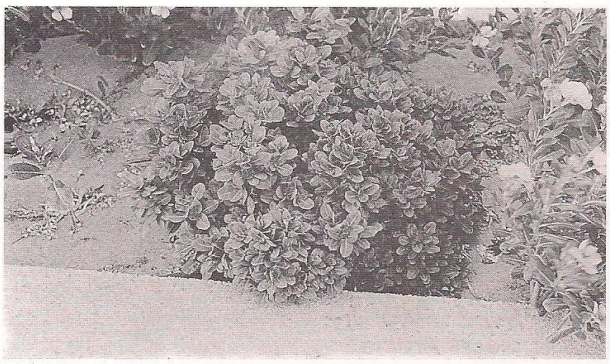

Figure 1. The variant plant that arose from gamma-irradiated seeds of normal *C. roseus* (L.) G. Don.

five explants of both shoot tips and axillary buds per treatment were placed individually in 25 x 150 mm culture tubes containing 15 ml of medium. Shoots developed from the initial explants were used for the subsequent multiplication stage.

SHOOT MULTIPLICATION AND ROOT INITIATION: Shoots, 2.0-2.5 cm long, were cultured in 98-mm culture vessels containing 40 ml of MS medium supplemented with 3.0% sucrose and various concentrations of BA (0.0, 1.0, 2.0, 3.0, or 4.0 mg L^{-1}) separately or in combination with NAA (0.0, 0.5, or 1.0 mg L^{-1}). Ten vessels were assigned per treatment, and all vessels were capped and then wrapped with Parafilm after subculture. The regenerated shoots (2.0-2.5 cm in length) were transferred to full or half strength, except iron, of MS basal salt medium supplemented with different concentrations of IAA, IBA, or NAA (0.0, 0.1, 0.5, 1.0, 2.0, or 3.0 mg L^{-1}) for root initiation. One microshoot was planted in each 25 x 150 mm culture tube containing 15 ml medium. Twelve tubes were assigned per treatment. All media were adjusted to pH 5.8 before the addition of 0.7% agar (Micro Agar, DUCHEF Biochemicals, The Netherlands) and autoclaved at 121°C for 20 min. All cultures were maintained at 25 $\pm$ 2°C under a 16-h light regime of 50-60 µmol m^{-2} s^{-1}, provided by cool-white fluorescent tubes.

Rooted microshoots were transferred into 10-cm pots filled with perlite and placed in a growth chamber (KBW 240, WTB Binder labortechnik Gmbh, Tuttlingen, Germany) for 3 weeks at 20 $\pm$ 2°C and 90% relative humidity (RH). The RH was decreased gradually for 2 weeks to 30-40%. The rooted microshoots were then kept in normal laboratory environment (25°C and 20-25% relative humidity) for 3 weeks before they were moved to a shade house. Data were recorded after 6 weeks of culture. Analysis of variance was employed to analyze the data and the Newman-Keuls multiple range test was performed to compare the means (TexasSoft, 1997).

Results

There were no significant differences among shoot tips and nodal segment explants. The presence of growth regulators induced shoot elongation and breaking of axillary buds. The number of newly-formed shoots increased threefold (6.2 shoots/explant) in the medium having growth regulators compared to growth regulator-free medium. The shoots were also taller (2.5-2.9 cm) than those of growth regulator-free medium (Table 1).

TABLE 1

Establishment media and explant type effects on bud breaking and shoot length of Catharanthus roseus in vitro.

Medium	Plant Part[1]	Shoot No.	Length of Shoot[2] (cm)
MS with[3], No GR	ST	1.92B[4]	1.63B
	NS	2.05B	1.70B
MS + 1.0 mg BA, 0.1 mg IBA & GA_3	ST	5.56A	2.49A
	NS	6.17A	2.91A

[1]ST = Shoot tips; NS = nodal segments.
[2]The length of the longest shoot was recorded in each replicate of each treatment.
[3]MS having no growth regulators (GR).
[4]Means bearing the same letters in the same column are not significantly different at the 5% level.

The highest number of shoot multiplication (15.1 shoots per shoot transferred) and the tallest shoot formed (7.0 cm) were induced by 1.0 mg L^{-1} BA in combination with 1.0 mg L^{-1} NAA (Table 2). However, there was no significant difference among this best combination and all remaining levels of BA with NAA 1.0 mg L^{-1} or BA at 3.0 or 4.0 mg L^{-1} with NAA 0.5 mg L^{-1}. Neither BA nor NAA alone were suitable to promote a high number of shoots. The concentration of MS basal salts had considerable effects on rooting percentage and root number irrespective of auxin type and concentration (Table 3). Most auxin levels gave over 82% rooting of shoots. NAA and IBA at 0.5 mgL^{-1} produced roots with

TABLE 2

Number of Catharanthus roseus in vitro shoots formed in response to different concentrations (mg L^{-1}) of BA and NAA.

NAA (mg L^{-1})	BA (mg L^{-1})	Shoot No.	Length of Shoot (cm)[1]
0.0	0.0	0.2G[2]	1.9E
	1.0	4.4EFG	2.5DE
	2.0	5.2DEF	2.3DE
	3.0	5.3DEF	2.3DE
	4.0	5.2DEF	2.5DE
0.5	0.0	0.9FG	2.4DE
	1.0	8.1CDE	4.0BC
	2.0	9.7BCD	3.5BCDE
	3.0	10.6ABC	3.3BCDE
	4.0	12.9AB	3.9BCD
1.0	0.0	0.7FG	3.1CDE
	1.0	15.1A	7.0A
	2.0	14.8A	4.7B
	3.0	10.9ABC	4.3BC
	4.0	10.6ABC	3.3BCDE

[1]The length of the longest shoot was recorded in each replicate of each treatment.
[2]Means bearing the same letters in the same column are not significantly different at the 5% level.

TABLE 3

Effects of half and full MS basal salt and auxins on rooting of Catharanthus roseus shoots in vitro.

Auxins	Concentration (mgL^{-1})	Half MS Salt			Full MS Salt		
		Root %	Root No.	Root Length[1] (cm)	Root %	Root No.	Root Length[1] (cm)
No auxin	0.0	55.6	1.77C[2]	0.37AB	16.7	1.08D	0.19E
IAA	0.1	91.7	7.00BC	0.61AB	100.0	6.75ABCD	0.98ABC
	0.5	100.0	6.25BC	0.98A	90.9	7.18ABC	1.06AB
	1.0	81.8	10.18ABC	0.67AB	76.9	4.85ABCD	0.56BCDE
	2.0	100.0	7.89BC	0.87AB	78.6	5.21ABCD	0.74BCDE
	3.0	90.9	10.46ABC	0.78AB	64.3	4.43ABCD	0.53BCDE
IBA	0.1	75.0	4.92BC	0.50AB	91.7	6.83ABCD	0.90ABCD
	0.5	100.0	7.33BC	0.93A	91.7	8.33AB	0.62BCDE
	1.0	100.0	12.25AB	1.13A	57.1	3.87ABCD	0.50BCDE
	2.0	100.0	10.09ABC	0.89AB	84.6	9.42A	0.59BCDE
	3.0	90.9	10.82ABC	0.83AB	69.2	3.15BCD	0.27E
NAA	0.1	91.7	7.83BC	0.84AB	91.7	5.17ABCD	1.38A
	0.5	100.0	18.00A	0.99A	91.7	6.08ABCD	0.43CDE
	1.0	100.0	17.42A	1.06A	75.0	5.50ABCD	0.28DE
	2.0	91.7	14.42AB	0.60AB	58.3	1.25D	0.32DE
	3.0	50.0	5.08BC	0.19BB	50.0	1.50CD	0.27DE

[1]The longest root was recorded in each replicate of each treatment.
[2]Means bearing the same letters in the same column are not significantly different by the Newman-Keuls multiple comparison test at the 5% level.

excellent root development (Figure 2). Whereas, high rooting percentage (90.9 - 100%) on normal MS salt occurred only at low levels of auxins, increasing auxin concentrations resulted in reduction in rooting percentage and root length. With both type of media, high levels of auxins, especially NAA, resulted in abnormal root morphology. The best root formation was observed on half MS salt containing 0.5 mg L^{-1} IBA or NAA (Table 3). Rooted microshoots successfully (95%) survived acclimatization *ex vitro*.

Discussion

Plant tissue culture has been applied for the rapid propagation and conservation of rare and endangered medicinal plants and other plant species (Krishnan *et al.*, 1995; Sudha and Seeni, 1996). Most tissue culture work on *C. roseus* has focused on alkaloid synthesis (Moreno *et al.*, 1995). The presence of growth regulators, especially cytokinins, in both establishment and multiplication media were essential for micropropagation of *C. roseus*. The balance between cytokinin and auxin in the multiplication medium was necessary to induce maximum shoot multiplication. BA and other cytokinins have been reported to be essential additives to tissue culture media of many plant species (Iapichino, 1996; Sudha *et al.*, 1998). Stapfer and Heuser (1985) found that BA was the best cytokinin for micropropagation of *Vinca minor* while BA alone at 14.5 mg L^{-1} was the optimal level (10.5 shoots/explant). In this study, BA alone was ineffective in producing multiple shoots and the optimal level that induced

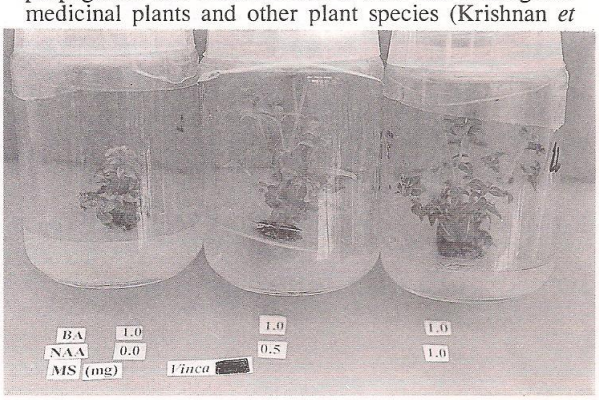

Figure 2. Multiple shoots of *C. roseus* (L.) G. Don. formed on MS medium.

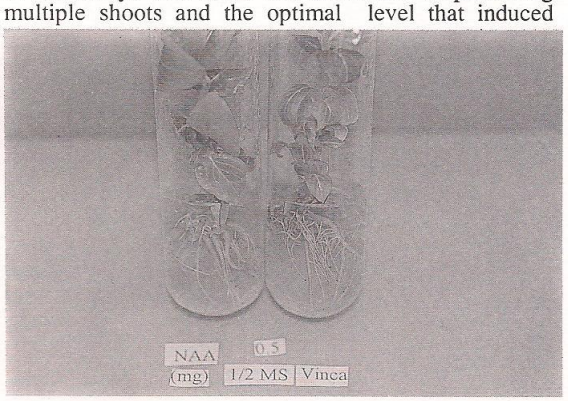

Figure 3. Rooted microshoots of *C. roseus* (L.) G. Don. on half strength of MS salt supplemented with 0.5 mg L^{-1} NAA.

maximum shoots (15.1 shoots/explant) was BA at 1.0 mg L^{-1} in combination with 1.0 mg L^{-1} NAA.

The salt concentration of MS medium had an obvious effect on the quality of root and rooting percentage. Half-strength MS salt has been reported to be better than full strength MS with other plant species (Atta-Alla and Van Staden, 1997; Al-Wasel, 1999). High levels of auxins inhibited or stimulated abnormal root formation (Zhang *et al.*, 1987; Dantu and Bhojwani, 1995). In this study, high auxin concentrations resulted in short and abnormal shoot morphology. Plants derived through tissue culture will be evaluated for their phenotypic stability and their alkaloid content.

Conclusion

The addition of growth regulators was essential to enhance the growth of the initial explants of periwinkle plant. The concentration and combination of BA and NAA were crucial to promote shoot multiplication and elongation. BA and NAA at 1.0 mg L^{-1} was the best combination for giving maximum shoot formation and better shoot elongation. Microshoots rooted better on half strength MS salt with 0.5 mg L^{-1} NAA or IBA. Thus, the tissue culture technique is a potential means for propagation and conservation of periwinkle and other elite plant species.

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