

***In vitro* culture of rose species (*Rosa* spp.) via axillary bud growth**

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ABSTRACT

The utilization of nodal stem cuttings containing dormant axillary buds as explants for plant production of two rose species; *Rosa chinensis* cv. 'Old Blush' and *R. centifolia* cv. 'Petite de Hollande' was demonstrated in this study. This propagation technique required the breaking of dormant axillary buds by aseptically culturing them in agar-solidified Woody Plant Medium (WPM) added with 0.5, 1.0, and 2.0 mg l⁻¹ of 6-benzylaminopurine (BAP) or, a combination of 2.0 mg l⁻¹ BAP and 0.01 mg l⁻¹ naphthalene acetic acid (NAA). Production of multiple adventitious shoots from one nodal stem explant was obtained after three months of culture in medium supplemented with 1.0 or 2.0 mg l⁻¹ BAP. Four types of plant morphology; single shoot (type 1), multiple shoots with normal leaves (type 2), cluster of tiny shoots with curly leaves (type 3), and single shoot with callus at the base (type 4) were observed from the axillary bud-derived plantlets. The rooting of plantlets was induced in WPM containing 0.25 to 1.0 mg l⁻¹ of indole-butyric acid (IBA) or, 2.0 mg l⁻¹ of indole-3-acetic acid (IAA).

Keywords: benzylamino purine, naphthalene acetic acid, nodal stem, woody plant medium

INTRODUCTION

Roses (*Rosa* spp.) represent a major commercial market for cutflowers, potted plants and garden bushes with over 200 M bushes planted worldwide each year (Short and Roberts, 1991 as cited by Marchant *et al.*, 1994). They are also rich sources of raw materials for cosmetic, medicinal and culinary purposes. Roses sold in flower shops and nurseries today are spontaneous or artificially-induced sports (mutations) and hybrids of varied parentage. These do not propagate easily, need sturdy well-rooting rootstock like *R. multiflora*, *R. canina*, and *R. corymbifera laxa* and require intensive cultivation. The old rose cultivars like *R. chinensis* cv. 'Old Blush' and *R. centifolia* cv. 'Petite de Hollande' are gaining back popularity because of their profound hardiness, resistance to pests and diseases and, ease in propagation that make them excellent donors of useful genes.

Plant tissue culture is an alternative to the traditional way of propagating roses thereby eliminating the use of rootstocks (Skirvin *et al.*, 1990), providing an effective worldwide distribution of rose plantlets at a reduced cost and approved by the international phytosanitary board (Schiva, 2001). This technique generates many plants from a selected/new genotype in a relatively short time and less space Reist (1985) as cited by Dubois *et al.* (1988), *in vitro* propagated roses sometimes yield more flowers than those propagated by cuttings. They are more compact and branch better when grown *ex vitro*. For highly heterozygous plants like roses, tissue culture via micropropagation provides stable clones where traditional breeding methods failed.

Reports of direct plant regeneration from axillary buds have been mainly on rose species such as *R. hybrida* (Miro, 1992; Ara *et al.*, 1997; Kim *et al.*, 2003). Likewise, more hybrid roses were used in studies involving biotechnological approaches compared to old cultivars (Khosh-Khui and Sink, 1982; Hsia and Korban, 1996). Thus, it is important to establish a plant regeneration system in old rose cultivars as requisite for their utilization in varietal improvement and germplasm conservation programs using biotechnological approaches such as genetic transformation and cryopreservation.

This study demonstrated the utilization of axillary buds of nodal stem cuttings to produce plantlets from two rose species, *R. chinensis* cv. 'Old Blush' and *R. centifolia* cv. 'Petite de Hollande'. The medium requirements during initial culture and subsequent plant production and rooting are presented.

MATERIALS AND METHODS

Care of donor plants

Mature plants of the two rose species, *Rosa chinensis* cv. 'Old Blush' and *R. centifolia* cv. 'Petite de Hollande' were used as source of nodal stem cuttings. Routine fertilization using complete fertilizer (16-16-16% of N, P₂O₅, K₂O) at 1 tbsp. per two gallons of water and, application of insecticide (Furadan, Bayer Phils. Inc.) at ~10 granules per plant were done once a month to obtain healthy and vigorous plants. Plants were constantly inspected for bugs and aphids and these were manually removed.

Culture medium and incubation condition

The basal woody plant medium (WPM) (Lloyd and McCown, 1981 as modified by Flynn *et al.*, 1990) containing myo-inositol (100 mg l⁻¹), nicotinic acid (0.5 mg l⁻¹), pyridoxine.HCl (0.5 mg l⁻¹), thiamine.HCl (0.7 mg l⁻¹), adenine sulphate (0.5 mg l⁻¹), L-leucine (0.4 mg l⁻¹), L-arginine (0.4 mg l⁻¹), L-tryptophan (0.2 mg l⁻¹), and glycine (2.0 mg l⁻¹), 30 g l⁻¹ refined white table sugar and solidified with 7 g l⁻¹ agar-agar (Pronadisa™, Hispanlab, S.A. Madrid) was used in all experiments. The pH of the medium was adjusted to 5.8 and then, the medium was dispensed at 10 ml in glass vials (95 x 28 mm) prior to autoclaving at 1.1 kg cm⁻² (121 °C) for 20 min. The culture condition was kept under 16h photoperiod provided two 40-W cool, white fluorescent tubes (Philips, TL40W/54/RS, Philippines) and 25±1°C unless, otherwise stated.

Experimental design and treatments

This experiment was laid out in a Completely Randomized Design (CRD). One-way and Univariate analysis of variance were used to analyze the data and Tukey's Honestly Significant Difference test (HSD) was applied to determine significant differences between treatment means.

Preparation of the nodal stem explants

Three to seven inches of apical stem cuttings containing not more than seven nodes were used as source of axillary buds. The stems were cut into segments containing two to three nodes and then surface sterilized by soaking

in 70% ethyl alcohol (ETOH) for 2 min followed by 20% commercial bleach solution (i.e., Zonrox) for 10 min. Finally, the stems were rinsed three to five times with sterile distilled water. Inside the laminar flow hood, the decontaminated stems were further cut into ~20 mm single node-stem pieces and inoculated into glass vial (95 mm × 28 mm) containing 5 ml of culture medium.

Regeneration of plantlets from axillary buds

The single-node stem segments were inoculated into agar-solidified WPM supplemented with BAP at a range of concentrations (0.5, 1.0, and 2.0 mg l⁻¹). The medium containing 2.0 mg l⁻¹ BAP and 0.01 mg l⁻¹ NAA reportedly effective for axillary bud culture of six hybrid tea cultivars (Kim et al., 2003) was included in the treatments for comparison purposes. The nodal stem cultures were incubated in dark at 25±1°C for 1 to 2 weeks to break the dormancy of axillary buds. Then, these were transferred to culture shelves with reduced light intensity for three weeks immediately followed by full light intensity for one week. The nodal stem cultures were subcultured once at 4 weeks interval to allow emergence of axillary buds. Fully emerged axillary buds were dissected from the nodal stem tissue to allow further growth and proliferation of adventitious shoots. Adventitious shoots bearing at least three to five leaves were individually separated and transferred to fresh culture medium and subcultured once at four weeks interval to allow the proliferation of shoots. The effects of the treatments were evaluated by observing the percentages of nodal stem segments showing tissue necrosis, emerging axillary buds and, shoot growth and development.

In vitro rooting of plantlets and acclimatization

The axillary bud-derived shoots (~10 mm in length) were transferred to WPM supplemented with combinations of indole-butyric acid (IBA) at 0.25, 0.5 and 1.0 mg l⁻¹, and indole-3-acetic acid (IAA) at 2.0 mg l⁻¹ to induce rooting. The medium containing 2.0 mg l⁻¹ IAA reportedly effective for rooting of shoots from several rose species (Ma et al., 1996) was included in the treatments for comparative purpose. The PGR-free medium served as control. Ten shoots were inoculated per treatment medium and incubated under 16 h photoperiod at 25±1 °C. After four weeks, the percentage of shoots producing roots, average length (mm) and number of roots were recorded. The plantlets

bearing at least three to five leaves and roots were taken out of the culture room and placed at room temperature (28 ± 2 °C) under 24 h photoperiod for one to two weeks for acclimatization. Thereafter, the plantlets were removed from the vials, washed of remaining agar and planted in clay pots containing a mixture of sterile vermiculite, compost, and garden soil at 1:1:1 (v/v) or, in Hoagland's nutrient solution (Hoagland and Arnon, 1938) and kept in the acclimatization area for four weeks. A glass cover was provided for each plantlet to simulate the high relative humidity of *in vitro* condition. After four weeks, the glass covers were removed. The percentage of survival of potted plants was gathered after three months of acclimatization.

RESULTS AND DISCUSSION

Regeneration of adventitious shoots from axillary buds

The viable axillary buds of nodal stem segments swelled and protruded while the non-viable buds gradually turned brown and died after two to three weeks of culture in agar-solidified WPM supplemented with BAP or, a combination of BAP and NAA. The viability of axillary buds was affected by the surface sterilization process whereas, the breaking of bud dormancy was influenced by BAP and NAA supplement in the culture medium. Generally, the emergence of axillary buds was observed from the two rose species after three weeks of culture in the BAP and NAA-supplemented medium and after seven weeks in the unsupplemented or control medium. The medium supplemented with 1.0 mg l^{-1} BAP (S_2) induced the highest percentage (80%) of emerging buds in *R. chinensis* cv. 'Old Blush' (Fig. 1). Likewise, the treatment medium (S_4) containing a combination of higher concentration of BAP (2.0 mg l^{-1}) and lower concentration of NAA (0.01 mg l^{-1}) induced 73.33% bud emergence in *R. centifolia* cv. 'Petite de Hollande'. On the contrary, the medium lacking plant growth regulators or, control (S_0) gave low percentage of emerging buds from *R. chinensis* cv. 'Old Blush' (43.33%) and *R. centifolia* cv. 'Petite de Hollande,' (40.00%) (Fig. 1). The importance of BAP in breaking the dormancy of buds of rosaceous plants like chestnut, apple, and cherry has been mentioned by Vieitez and Vieitez (1980). BAP has also been found effective for axillary bud growth of tea plants (Nakamura, 1991); *R. hybrida* L. cv. 'Forever Yours' (Wilkowske, 1981) and cv. 'Peace'

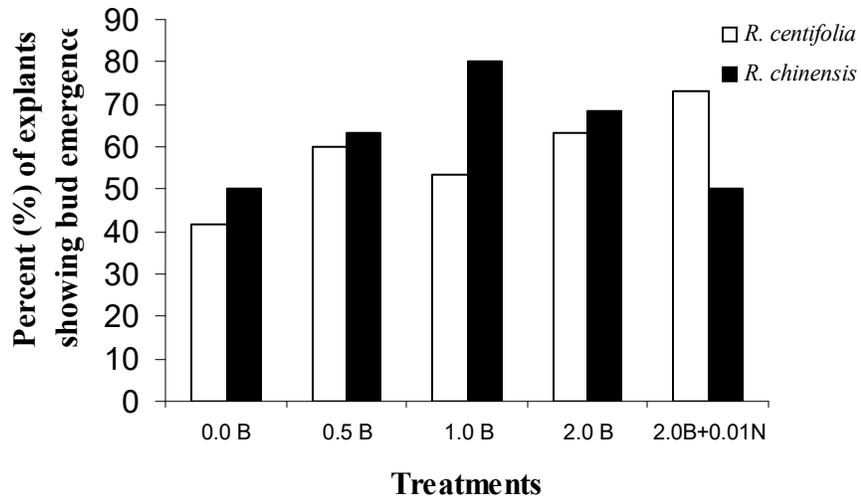


Figure 1. Percentage of nodal stem explants of *Rosa centifolia* cv. 'Petite de Hollande' and *R. chinensis* cv. 'Old Blush' showing emergence of axillary buds after 3 weeks of culture in agar-solidified WPM added with BAP (B) or, BAP and NAA (N) in mg l^{-1} .

(Ara *et al.*, 1997); and several rose species (Ma *et al.*, 1996; Roy *et al.*, 2004) except, in *R. chinensis* and *R. centifolia*. Thus, this paper reports for the first time the effects of BAP on *R. chinensis* and *R. centifolia* cultivars.

In this study, four types of plant morphology of *R. chinensis* and *R. centifolia* cultivars were observed depending on the culture medium and plant growth regulator supplement; (1) type 1 - single shoot, (2) type 2 - multiple shoots with normal leaves, (3) type 3 - cluster of tiny shoots with curly leaves, and (4) type 4 - single shoot with callus at the base (Fig. 2). The type 1 shoot was produced through the growth of the axillary bud in plant growth regulator-free WPM or, control medium (S_0). The type 2 shoots was obtained in medium supplemented with 1.0 mg l⁻¹ BAP (S_2) and 2.0 mg l⁻¹ BAP (S_3). The type 3 shoots was produced in medium containing 2.0 mg l⁻¹ BAP (S_3) and, a combination of 2.0 mg l⁻¹ BAP and 0.01 mg l⁻¹ NAA (S_4). Production of multiple adventitious shoots in BAP-containing medium agrees with the report of Kim *et al.* (2003). This is further supported by the findings of Huth (1978) as cited by Vieitez *et al.* (1983) that culture medium containing a combination of NAA and BAP was effective for shoot multiplication of apples.

An average of 1.23 to 1.87 adventitious shoots was produced from both rose species four weeks after inoculation of nodal stem segments in the agar-solidified WPM supplemented with BAP and NAA (Table 1). Four weeks after subculture (or eight weeks after explant inoculation), the average shoot production per shoot was increased to 1.33-2.17 indicating the proliferation of adventitious shoots in the BAP and NAA-supplemented media. The non-supplemented medium or, control (S_0) supported the growth of the existing shoot meristem of the axillary bud during the initial culture but did not induce shoot proliferation after subculture except, in *R. chinensis* cv. 'Old Bush'. All averages however, were not significantly different from each other indicating that the range of BAP and NAA concentrations incorporated in the medium had comparable effects.

The production of callus at the base of type 4 shoot was caused by high BAP concentration and/or the presence of NAA (Chawla, 2002) in the culture medium. The calli however, did not interfere with the growth and development of the shoot. The calli were non-regenerable hence, these were detached from the shoots during subculture. Callus formation of tissue cultured shoots has been observed in nodal cultures of *Santalum* species (Barlass *et al.*, 1980 as cited by Vieitez *et al.*, 1983), and from two rose species and hybrid cultivars treated with NAA and BAP combinations (Khosh-Khui and Sink, 1982).

Table 1. Number of adventitious shoots from two rose species after 4 weeks of culture in agar-solidified WPM containing BAP (B) and NAA (N) in mg l⁻¹

Rose Genotype	Plant growth regulator in mg l ⁻¹	Ave. number of shoots after 4 weeks	Ave. number of shoots after 8* weeks
<i>Rosa centifolia</i> cv. 'Petite de Hollande'	S ₀ -0.0 B	1.00	1.00
	S ₁ -0.5 B	1.40	1.33
	S ₂ -1.0 B	1.87	2.17
	S ₃ -2.0 B	1.63	2.13
	S ₄ -2.0 B+ 0.01 N	1.70	1.87
<i>Rosa chinensis</i> cv. 'Old Blush'	S ₀ -0.0 B	1.00	1.20
	S ₁ -0.5 B	1.23	1.87
	S ₂ -1.0 B	1.53	1.80
	S ₃ -2.0 B	1.87	2.00
	S ₄ -2.0 B	1.57	1.68
	+ 0.01 N*		

*4 weeks after subculture of adventitious shoots into fresh medium

In vitro rooting of plantlets

Axillary bud-derived plantlets (~1.0 in. in length) bearing at least five to seven leaves produced two to three roots after two to three weeks of culture in agar-solidified WPM containing IBA (Fig. 3A, B) or IAA and, after eight weeks in plant growth regulator-free medium. The roots were white and actively growing, but gradually turned brown and stopped growing. After three weeks, the roots resumed their growth, increased in length and produced lateral roots. The plantlets also produced non-embryogenic calli at the base due to the presence of IBA and IAA in the rooting medium. These calli did not affect the rooting process and were removed during subculture of the plantlets.

The addition of IBA and IAA in the rooting medium induced the production of roots from the axillary bud-derived plantlets compared to the control or medium lacking IBA or IAA (Table 2). The average number of roots of plantlets cultured in the five treatment media was comparable. However, the addition of 0.25 mg l⁻¹ IBA was found to be more effective since it induced root elongation (Table 2). The result also indicated that plantlets root readily in medium containing lower IBA concentration as reported in several rose species (Norton and Boe, 1982; Chatani *et al.*, 1996; Ma *et al.*, 1996; Ara *et al.*, 1997; Mohan and Ibrahim, 2000).

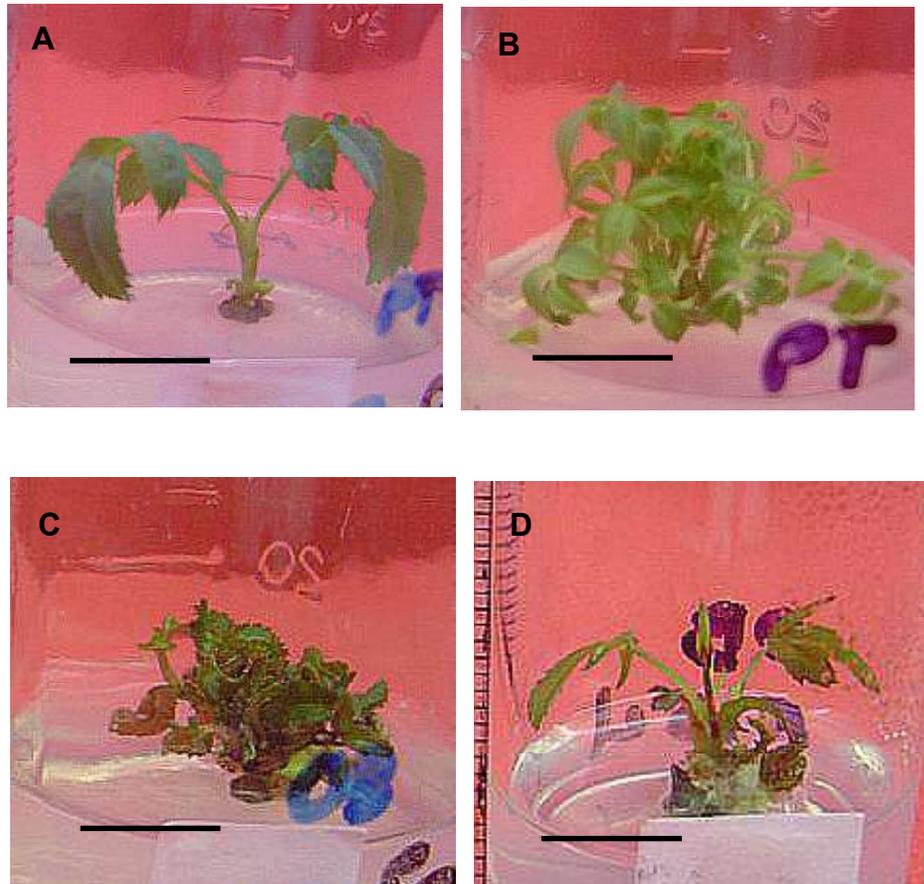


Figure 2. Four types of plant morphology from axillary bud-derived plantlets of *Rosa chinensis* cv. 'Old Blush.' and *R. centifolia* cv. 'Petite de Hollande'. (A) single shoot, (B) multiple shoots, (C) a cluster of tiny shoots with curly leaves, and (D) single shoot with callus; (A & B, bar = 0.7 in.; D & C, bar = 0.5 in.).

Table 2. *In vitro* rooting of axillary bud-derived shoots of rose species after 4 weeks of culture in agar-solidified WPM medium containing indole butyric acid (IBA) or indole acetic acid (IAA) in mg l⁻¹

Plant growth regulator(mg l ⁻¹)	Average number of roots	Average length of roots (mm)
R ₀ – 0.0	0.00b ¹	nt ²
R ₁ – 0.25 IBA	3.25a	11.64b
R ₂ – 0.50 IBA	2.50ab	8.42b
R ₃ – 1.00 IBA	3.55a	8.36b
R ₄ – 2.00 IAA ³	1.35ab	21.78 ^a

¹Means followed by the same letter within a column are not significantly different from each other at 5 % level, Tukey HSD; ²not tested; ³based on Ma *et al.* (1996)

Recovery and acclimatization of plantlets

The regenerated plantlets bearing at least five roots and five to eight leaves survived the acclimatization period under ordinary room condition. However, the plantlets showed variable growth response after one to two months of transfer in Hoagland's nutrient solution (Fig. 3C) and in sterile potting medium (vermiculite, compost, and garden soil at 1:1:1; v/v) (Fig. 3D). The transplanted plantlets initially survived under *ex vitro* condition using the two substrates. However, after one to two months, the plantlets wilted and died. The poor establishment of plantlets in soil and in Hoagland's solution could be due to inefficient functioning of the roots produced during *in vitro* culture (Conger, 1981). The roots formed *de novo* were poorly attached to the vascular system of the shoot due to a close system formed by the xylem tissue across the shoot base prior to root formation. The plantlets also failed to form new roots in soil and nutrient solution. Thus, the conditions needed to enhance rooting and establishment of plantlets in soil need to be further investigated.

CONCLUSION

Culturing nodal stem segments in agar-solidified WPM containing BAP and NAA induced the breaking of dormancy of axillary buds of *Rosa chinensis* cv. 'Old Blush' and *R. centifolia* cv. "Petite de Hollande". Four types of plant morphology were observed from the axillary bud-derived shoots

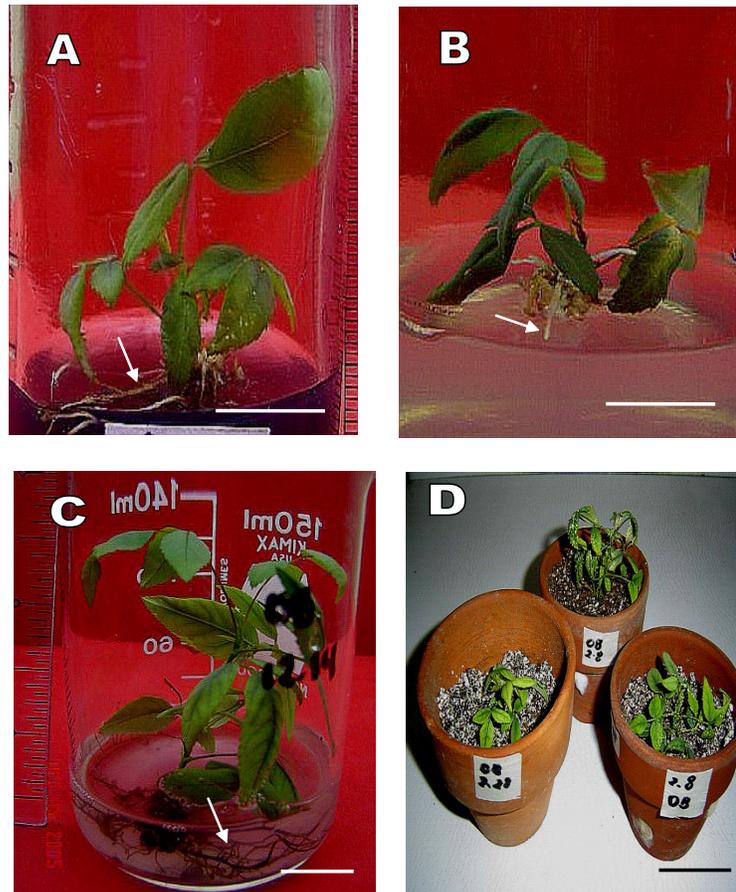


Figure 3. Rooting of *Rosa centifolia* cv. 'Petite de Hollande' and *R. chinensis* cv. 'Old Blush' plantlets in agar-solidified WPM containing 0.25 mg l⁻¹ IBA (A, B) and in Hoagland solution (C) prior to potting the rooted plantlets in soil (D). (A-C, bar 1.0 cm; D, bar 1.0 in; arrows indicate adventitious roots)

depending on the BAP and NAA supplement in the culture medium. The medium supplemented with 1.0 mg l⁻¹ BAP or, 2.0 mg l⁻¹ BAP and 0.01 mg l⁻¹ NAA effectively resulted in the production of multiple shoots from both rose species. The medium containing 0.25 mg l⁻¹ IBA was sufficient to initiate rooting of shoots under *in vitro* condition. Further study is needed to increase the survival of plantlets transplanted in soil.

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