A protocol for mass production of *Rosa hybrida* cv. Iceberg through *in vitro* propagation

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Abstract

Interactive effect of plant growth regulators 6-Benzylaminopurine (BAP) (0, 2, 4 and 8 μM) and 1-Naphtalene acetic acid (NAA) (0, 0.05, 0.25 and 0.5 μM) in Van der Salm (VS) medium was used to optimize *in vitro* propagation of *Rosa hybrida* cv. Iceberg. Shoot proliferation and number of new leaves were measured as growth indicators. As the concentration of BAP was raised, growth rate increased with all of the above NAA concentrations. However, the highest number of axillary shoots and new leaves were produced with 4 μM BAP, which was considered the optimal level. A multiplication rate of 10 folds with a maximum number of axillary shoots (10.1) and new leaves per explant (25) were obtained in the medium containing 4 μM BAP plus 0.5 μM NAA. *In vitro*-derived shoots were used to investigate root initiation and growth by lowering the concentration of VS mineral salts and vitamins. Three strengths of VS (full, 1/2 and 1/4) were compared in semi-solid and liquid medium. The average number of roots (4.35) and root length (0.82 cm) were significantly higher in 1/4 strength VS. The highest percentage of rooting (93.33%) and number of roots (4.45) were significantly higher in semi-solid than liquid medium. The regenerated plantlets were successfully transferred to soil and the survival rates of the rooted plantlets transferred to soil were 70% and 90% in plants treated with semi-sold and liquid media, respectively.

Keywords**:** *In vitro* propagation; Root initiation; *Rosa hybrida*; Shoot proliferation.

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INTRODUCTION

Roses are the most economically important flowers in the world. There are more than 20,000 commercial cultivars, which are collectively based on only 8 of the approximately 200 wild species in the genus *rosa* (Roberts and Smith, 1990).

Traditionally, most ornamental roses are heterozygous and do not breed true to type, they are therefore propagated vegetatively. Miniature roses are usually propagated by cuttings but the roses are usually propagated by budding or bench-grafting onto rootstocks of species such as *Rosa. canina* 'Inermis' and *Rosa multiflora* 'Simplex' (Short and Roberts, 1991). The conventional propagating methods are very slow, time consuming, and tiring. Tissue culture on the other hand is becoming increasingly popular as an alternative means of plant vegetative propagation.

Although the presence of a cytokinin is almost always advantageous, and is often all that is required, optimum rates of shoot initiation generally occur with combinations of auxin and cytokinin (George, 1993). Natural and synthetic auxins have been used extensively *in vitro* in plant cell tissue and organ culture to obtain specific morphogenic responses. One of the most important applications of auxins is the induction of adventitious root formation. Induction of adventitious roots in roses has been demonstrated by workers such as Skirvin *et al.* (1990). But the presence of auxin in defined combinations with cytokinins in the culture medium is also necessary to obtain adventitious shoot formation (Caboni and Tonelli, 1999).

'Iceberg', also known as 'Fée des Neiges', a repeat reblooming floribunda, was bred by Kordes in Germany and is the result of a cross between 'Robin Hood', a Pemberton bred hybrid musk (1927) and 'Virgo' a large flowered hybrid tea rose (1947). It's ever present double white flowers, often with a flush of pink in spring and fall, are lightly fragrant. This rose has won many awards including the National Rose Society Gold Medal in 1958, the Baden-Baden Gold Medal in 1958, the ADR Anerkannte Deutsche Rose (Germany) in 1960, the World's Favorite Rose in 1983 and the Royal Horticulture Society Award of Garden Merit in 1993 (Figure 1a).

Although tissue culture of roses has been reported by many authors but procedures for mass production at commercial level has not been reported in the scientific publications. In the present study, attempts were made to assess the interactive influence of BAP (6-Benzylaminopurine) and NAA (1-Naphtalene acetic acid) concentrations on the growth rate of *Rosa hybrida* cv. Iceberg for the first time, and thus increase growth rate to a level suitable for commercial use. We also describe a procedure for root initiation, root growth and acclimatization of plantlets to *in vivo* conditions.

Figure 1. a) Iceberg. **b**) Shoot proliferation **c**) From left to right; shoots rooted in three strengths of VS medium (full, 1/2 and 1/4) and control (water) **d**) Shoots rooted in sorbarods.

MATERIALS AND METHODS

Plant material and general procedures: Nodal segments (1-1.5 cm) were taken from the stems of 'Iceberg' plants in the rose garden of the Agricultural Biotechnology Research Institute of Iran (ABRII). They were washed thoroughly with running tap water for half an hour and surface sterilized for 30 seconds in 70% (v/v) ethanol, followed by a 15 min soak in 2.5% (v/v) sodium hypochlorite solution with a few drops of Tween-20 as a wetting agent, and then rinsed three times with sterile distilled water. MS (Murashige and Skoog, 1962) basal medium (without hormone) was used for the *in vitro* of induction of explants in culture; the pH of the medium was adjusted to 5.8 before adding 8 gl^{-1} plant agar. Media were autoclaved for 15 min at 121ºC and 1.2 kPa pressure. Cultures were placed under high pressure metal halide lamps on a 16/8 hour light/dark cycle in a culture room maintained at 21 ± 1ºC. Axillary shoots were detached and transferred to VS medium (van der Salm *et al*., 1994) in which FeNaEDTA was replaced by FeEDDHA as iron source after 14 days.

Shoot proliferation: The shoot proliferation media contained full strength VS salts and vitamins with various levels of BAP (0, 2, 4 and 8 µM) in combination with NAA (0, 0.05, 0.25 and 0.5 µM). Each treatment involved 5 repeats with 5 explants (25 explants). Number of axillary shoots and number of new leaves were recorded after 21 days for three subsequent subcultures and the averages were calculated.

Root initiation: Shoots were cultured on shoot elongation medium (VS mineral salts and vitamins without hormones) for 21 days prior to rooting treatments. For rooting, three concentrations of VS mineral salts and vitamins (full-, half-, and quarter-strength) containing IBA (3-Indolebutyric acid) (0.25 µM) and NAA (0.25 µM) were tested in semi-solid and liquid media. For liquid medium, sorbarods (Cellulose support plugs; Sorbarod, Ilacon, UK) were used (Figure 1d). Each treatment involved 5 repeats with 5 explants (25 explants). After 21 days, number of roots and their lengths were recorded and data for different concentrations of VS media (full, 1/2 and 1/4) and state of media (semi-sold and liquid) were recorded.

Transfer to soil: *In vitro* rooted plantlets either in semi-solid medium or in sorbarods containing liquid medium were taken out from the jars and gently washed under running water and then transferred to plastic cups containing a mixture of pit and perlite (1:1) each covered with a transparent plastic cup. A hole was made in the covering cup every day, until day

Table 1. Interactive effect of different concentrations of growth regulators (BAP and NAA) on shoot proliferation and number of new leaves in 'Iceberg'. **a**) Average number of axillary shoots per explant, **b**) Average number of new leaves produced per explant. Different letters show significant differences according to Duncan's Multiple Range Test ($P \leq 0.05$).

BAP (μM) / NAA (μM)	0	2	4	8
0.00	0.00^{c}	8.90^{ab}	8.60^{ab}	8.25^{ab}
0.05	0.65^{c}	8.85^{ab}	9.15^{a}	6.85^{ab}
0.25	1.00^{c}	8.80^{ab}	7.25^{ab}	4.25^{b}
0.50	0.85^{c}	8.55^{ab}	10.1^{a}	7.25^{ab}

a

BAP (μM) / NAA (μM)	0	2	4	8
0.00	5.65^{c}	16.4^{b}	21.0^{ab}	21.9ab
0.05	7.65^{c}	21.7^{ab}	20.1^{ab}	16.5ab
0.25	7.60^{c}	17.4^{ab}	16.1^{b}	15.3b
0.50	6.10^{c}	21.4^{ab}	25.0^{a}	18.2ab

b

10, when the cover was removed completely. Plantlets were treated with NPK (nitrogen-phosphate-potassium) (7 g/l) fertilizer every 14 days and the percentage of survivors was recorded after 60 days.

Experimental design and statistical analysis: Shoot proliferation experiment was analyzed in a factorial based completely random design and root initiation experiment was as a completely random design. Each experiment was repeated twice. Analysis of variance was performed and comparisons of means were conducted using Duncan's Multiple Range Test. All analyses were regarded as significant at $P \leq 0.05$.

RESULTS

Shoot proliferation: Micropropagation of rose cultivars range from easy to difficult; however multiplication rate (axillary shoots and new leaf production) of *R. hybrida* cv. Iceberg was occasionally high, up to 13 shoots and 34 leaves (Figure 1b), depending on the cytokinin content of the medium. Results presented in Table 1(a, b) indicate that when 4 μM BAP was used in combination with 0.05 and 0.5 μM NAA, significant differences were not observed in the average number of axillary shoots, but there was a significant difference in the average number of new leaves produced. A multiplication rate of 10 fold with an average number of axillary shoots (10.1) and new leaves per explant (25) were obtained in the medium containing 4 μM BAP plus 0.5 μM NAA.

The results of the present study demonstrated that as the concentration of BAP was increased the number of axillary shoots and new leaves per explant were increased. Although in some cases the significant differences in the increase of growth rates were not apparent, but it was statistically established that the maximum growth rate (number of axillary shoots and new leaves per explant) was achieved when 4 μM of BAP and 0.50 μM of NAA were used. As the concentration of BAP was raised to 8 μM, a reduced growth rate was noted with all of the NAA concentrations, (Table 1).

Root initiation and acclimatization of *in vitro* plantlets: Figure 1c illustrates the morphogenetic responses of the shoots treated with three (full, 1/2 and 1/4) strengths of VS salts and vitamins. The results indicate that the average percentage of rooting was higher in 1/4 strength VS (85%), although it was not significantly different from the plants treated with 1/2 VS (Table 2). The average number of roots (4.35) and root length (0.82 cm) were significantly higher in 1/4 strength VS.

Table 2 compares the effect of semi-solid and liquid media. Statistical analysis indicates that there was not a significant difference between the average root length in semi-solid and liquid media. However, the highest percentage of rooting (93.33%) and number of roots (4.45) were observed in the semi-solid medium. The survival rates of the rooted plantlets transferred to soil were 70% and 90% in plants treated with semi-sold and liquid media, respectively.

Table 2. Comparing average percentage of rooting, number of roots produced and root length in different concentrations of VS (full, 1/2 and 1/4) salts and vitamins and semi-solid and liquid media. Means in each column with different letters show significant differences according to Duncan's Multiple Range Test ($P \leq 0.05$).

Concentration	Rooting (%)	Number of roots/explant	Root length (cm)
VS	57.5^{b}	2.05^{c}	0.34^{b}
1/2 VS	72.5^{a}	3.20^{b}	0.50^{b}
1/4 VS	85.0^{a}	4.35^{a}	0.82^{a}
Semi-solid medium	93.33^{a}	4.45^{a}	0.55^{a}
Liquid medium	50.00^{b}	1.95^{b}	0.56^{a}

DISCUSSION

The choice of explant for initiation of culture is largely dictated by the method to be adopted for *in vitro* propagation. Explants with vegetative meristems are often suitable for enhanced axillary branching. The most commonly used explant in shoot proliferation of roses is the nodal stem segment, wherein the axillary bud is made to proliferate and form multiple shoots. The performance of nodal segments is much better than the shoot tips (Horn, 1992).

In the present study, the highest multiplication rate of 10 fold was obtained in the medium containing BAP (1.0-10.0 mg l^{-1}) which was essential for bud break and shoot multiplication of *R. hybrida*. Pati *et al.* (2001) optimized conditions by using a BAP concentration of 5 μM for shoot proliferation in *Rosa damascena* and *Rosa bourboniana*. Bressan *et al.* (1982) reported maximum promotive effects using BAP as compared with 2-isopentyladenine (2-ip). Rout *et al.* (1990) reported that the presence of cytokinin in the culture medium helped in the year round multiplication of shoots in hybrid roses. Roberts and Schum (2003) indicated that for introduction into culture and multiplication, BAP at a concentration of 2 μM was adequate for most species and cultivars of rose. They reported that a maximum multiplication of two to five folds every 4-6 weeks is possible for most rose species and cultivars, although using the mother plant method increased the multiplication rate by an average of 28 fold.

Variation in growth rate (number of axillary shoots and new leaves per explant) was noted with all of the NAA concentrations when the BAP concentration was changed, suggesting that increasing BAP levels were more effective in stimulating growth rate than changing the NAA levels. This is in accordance with Vijaya *et al.* (1991), who reported that BAP was the most effective growth regulator in stimulating shoot proliferation. Kim *et al.* (2003) obtained the best shoot proliferation in the present of 2 mg l^{-1} of BAP and 0.01 mg l^{-1} of NAA in full-strength MS salts. They also showed that *in vitro* shoot proliferation and multiplication are largely based on media formulations containing cytokinins as a major plant growth regenators, whereas, in some cases, low concentrations of auxins and gibberellic acid 3 (GA_3) were also used. Statistical analysis of our data showed that a moderate BAP level (4 μM) combined with 0.5 μM NAA resulted in the highest growth rate, which is achieved only when the concentrations of endogenous or exogenous auxins and cytokinins are balanced (George, 1993).

Although rose shoots often proliferate readily *in vitro*, rooting of those shoots is proved to be more difficult. Kim *et al.* (2003) suggested that rooting is affected by genotype; MS medium salts concentration, cold dark treatment, and auxin type. The average number of roots and root length were significantly higher in 1/4 strength VS medium which is in accordance with Skirvin *et al.* (1990) who reported that the reduced salt concentration generally increased rooting in MS medium. Faisal and Al-Amin (2000) also reported that using 0.2 mg l^{-1} of IBA and 0.2 mg l^{-1} of IAA with half strength MS were the best combination for root formation in *Chrysanthemum morifolium.* Enhanced root initiation and growth in 1/4 strength medium could be attributed to a more favorable nitrogen concentration availability and thus a higher rate of rhizogenesis than provided by full VS mineral salts (Hyndman *et al*., 1982).

Our investigation showed that the highest percentage of rooting and number of roots were observed in semi-solid medium. Ebrahim and Ibrahim (2000) also reported that solid medium supported the fastest growth and development of both roots and shoots. Ghashghaie *et al.* (1991) showed that in liquid medium, vitrification was a response to osmotic shock, and therefore reduced rate of rooting, although in the present investigation due to the use of sorbarods, vitrification was not observed but our results also showed that lower percentage of rooting and number of roots were observed in plants treated with liquid medium. Rout *et al.* (1990) also reported that rooting of microshoots was better in solid medium than that in liquid medium too.

The survival rate of the rooted plantlets transferred to soil was higher in plantlets treated in liquid medium than the ones treated with semi-sold medium. Roberts *et al.* (1990) reported that after transfer to plastic cups, plantlets that were rooted in sorbarod plugs wilted less but transpired more water than plantlets taken from agar. The protective environment of the sorbarods may have contributed to less damaged roots, higher uptake of water and therefore higher survival rates.

Acknowledgments

This work was funded by the Agricultural Biotechnology Research Institute of Iran (ABRII) (Project number: 2-013-140000-01-0000-84004).

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