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Optimization of Factors Affecting *in vitro* Proliferation and Rooting of *Rosa hybrida* L. cv. 'Rafaela'

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Abstract: *Rosa hybrida* L. cv. 'Rafaela' was successfully micropropagated through auxiliary buds. The explants were cultured on Murashige and Skoog (MS) medium supplemented with Benzyl adenin (BA) (0 to 12 mg L^{-1}) and agar (4.4 and 5.6 g L^{-1}). BA significantly affected the number and height of produced shoots. The highest proliferation rate (4.78) was achieved on MS medium containing 8 mg L^{-1} BA and 5.6 g L^{-1} agar. The effect of agar was significant only on the height of shoots. The effects of different concentrations of IAA (0 to 6 mg L^{-1}) and MS salt concentration (1/4, ½ and 3/4) were also examined for optimal rooting. The results showed that the root number was significantly increased in higher concentrations of IAA but salt concentration had a little effect on rooting parameters. Rooted plantlets were transferred to 3 different mixtures of sterile soil consisting of peat moss alone, peat moss + sand 1:1 (v/v) and perlite + vermiculite 1:1 (v/v) and after 4 weeks acclimatization, were placed in the greenhouse.

Key words: Micropropagation, rooting, rose, shoot proliferation

INTRODUCTION

Roses (*Rosa hybrida* L.) are one of the most important commercial crops grown for a variety of purposes such as pot plant, garden plant and cut flower production. In spite of the numerous investigations on different aspects of *in vitro* culture of this genus, still more studies are needed to optimize protocols for micropropagation of a specific desired cultivar. High heterozygosity present in this genus is partly responsible for this necessity. Therefore, some adjustments for a specific protocol for *in vitro* culture of different cultivars need further investigation. Micropropagation of roses has the potential to be commercialized and is now practiced on a large scale in some countries because it is a very rapid, all-year-round method of propagation especially advantageous for multiplication of new cultivars. Furthermore, production of healthy and disease-free plants has the advantages associated with health certification of *in vitro* plants for export (Khosh-Khui and Teixeira da Silva, 2006). The cultivar 'Rafaela' has the potential for being cultured in Iran and the cut flowers being exported to other countries.

In vitro culture of roses can be performed using auxiliary buds as explants (Khosh-Khui and Sink, 1982a; Arnold *et al*., 1995; Marcelis van Acker and Scholten, 1995; Gudin, 2001). Different factors like growth regulators, explant and agar are effective on auxiliary bud culture (Rout *et al*., 1999, Gudin, 2001). *In vitro* shoot proliferation and multiplication are largely based on media formulations containing cytokinins as a major plant growth regulator, whereas, in some cases, low concentrations of auxin and GA_3 were also used (He *et al*., 1996; Syamal and Singh, 1996; Sahoo and Debata, 1997; Singh and Syamal, 1999; Kumar *et al*., 2001; Carelli and Echeverrigaray, 2002).

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Inclusion of BA in the culture medium was promoted a higher number of shoots per explant compared with Kin and 2ip (Carelli and Echeverrigaray, 2002). In an experiment with *R. damascena* and *R. bourboniana* (Pati *et al*., 2006), the quantity of liquid medium was found to play an important role in shoot growth and multiplication which completely eliminated the possibility of vitrification and facilitated better quality shoots in terms of shoot length and thickness as compared to gelled medium.

Arnold *et al*. (1995) reported that microshoots of *Rosa kordesii* cultivars 'Jchin Fanklin' and 'Champlain' rooted normally in MS medium with low or no auxin. Optimum rooting in cultivar 'Champlain' was achieved at a high concentration of IAA with low concentration of salt or intermediate concentration of IBA and NAA with low to medium concentrations of salt. Excised shoots of *R. hybrida* L. were rooted well on ½ MS free hormone medium (Ibrahim and Debergh, 2001).

The major objective of this study was to micropropagate the cultivar 'Rafaela' which is an important cultivar of roses in Iran and optimizing the hormones, agar and mineral salts levels for proliferation and rooting and also finding the best soil mixture for acclimatization of this cultivar.

MATERIALS AND METHODS

Plant Material

In early October 2004, single undeveloped axillary buds were excised from the middle part of vegetative shoots of greenhouse-grown cultivar 'Rafaela' of roses. This study was conducted at Department of Biotechnology, National Research Center of Ornamental Plants, Mahallat, Iran.

Establishment Stage

Single nodes with 1.5-2 cm long were rinsed for 20 min with water containing commercial dish washer solution (Golrang 0.1%) and then were sterilized with 2% (w/v) sodium hypochlorite for 20 min followed by at least three times rinsing with distilled autoclaved water. Explants, 10 mm long, were placed in 21×180 mm^2 tubes containing 15 mL of MS (Murashige and Skoog, 1962) medium supplemented with 12 different combinations of agar (4.4 and 5.6 g L^{-1}) and BA (0, 0.8, 1.6, 4, 8 and 12 mg L^{-1}), 30 g L^{-1} sucrose and 0.5 g L^{-1} charcoal. The pH of media was adjusted to 5.7 prior to adding agar. Cultures were kept under a 16 h photoperiod of 2500 lux light intensity at 25±2°C.

The percentages of developed shoots, number of shoots per explant and the length of shoot per explant were recorded.

Shoot Multiplication

After 25 days, shoots (with average length 3.92±0.4 cm) were excised from establishment media and cultured in 300 mL glass bottles containing 25 mL of shoot multiplication media. MS medium supplemented with agar (4.4 and 5.6 g L^{-1}) and BA (0, 0.8, 1.6, 4, 8 and 12 mg L^{-1}). Totally, 12 combinations were considered for multiplication media. The pH was adjusted to 5.7 prior to adding agar. The number of shoot per explant, the length of shoot per explant and the number of leave per shoot were recorded.

Rooting and Acclimatization

Shoots with an average length of 3.45±0.3 cm and with an average number of leaves 5.89±0.9 were transferred to the rooting media. For rooting, media with different concentrations of MS salts 1/4, ½ and 3/4 along with IAA at 4 levels (0, 1.5, 3 and 6 mg L^{-1}) were used. The pH was adjusted on 5.7 prior to adding 7 g L^{-1} agar.

After 25 days the percentage of rooting, the number of root in every shoot and the length of root were recorded. Rooted plantlets were transferred to 3 different soil mixture containing sterile soil consisting of peat moss alone, peat moss + sand 1:1 (v/v) and perlite + vermiculite 1:1 (v/v) and after

4 weeks acclimatization, were placed in the greenhouse. First the plantlets were covered tightly in greenhouse trays and later the covers were gradually removed.

Statistical Analysis

All experiments were conducted as a completely randomized design with 3 replications and 10 samples for each treatment. Data were statistically analyzed and the means were compared using Duncan's new multiple range test (DNMRT).

RESULTS AND DISCUSSION

Establishment Stage

Using single node section was successful for proliferation (Fig. 1A). Application of different concentrations of BA and agar did not significantly affect the percentage of developed shoots, the number of shoots and shoot length (data are not shown).

Shoot Multiplication

The effect of BA on number of developed shoots was significant at 1% level but the effects of agar and the interaction between BA and agar was not significant. The result showed that BA is necessary for proliferation (Fig. 1B). This is in agreement with the results obtained by other investigators on different species and cultivars (Hasegawa, 1980; Mederos and Enriquez, 1987; Valles and Boxus, 1987; Rout *et al*., 1999).

The number of shoots was increased with increasing concentration of BA in the media, but shoot elongation was decreased. In the absence of BA (data are not shown), all shoots died within 2 weeks.

Fig. 1: (A) Growth of shoot from single node of cv. 'Rafaela' after 25 days of culture (B) Shoot proliferation in MS medium supplemented with 5.6 g L^{-1} agar and 8 mg L^{-1} BA 6 weeks after culture (C) Plantlets successfully acclimatized to *ex vitro* conditions 4 weeks after transfer and (D) Plants grown in greenhouse 12 weeks after transfer

Table 1: Effects of various concentrations of agar and BA on axillary shoot proliferation of rose cultivar 'Rafaela' 45 days after culture on MS medium

Agar (g L^{-1})	BA (mg L^{-1})	No. of shoots	Shoot length (cm)	Leaf No.
4.4	0.8†	1.05e	4.04a	6.62a
	1.6	1.89de	3.89a	5.99ab
	4.0	3.50bc	3.36abc	5.72abc
	8.0	3.83b	3.27abc	5.61abc
	12.0	3.76b	2.87bcd	4.70c
5.6	0.8	1.30e	3.93a	5.69abc
	1.6	2.67cd	3.49ab	5.51bc
	4.0	3.40bc	2.72bcd	5.32bc
	8.0	4.78a	2.48cd	5.37bc
	12.0	3.75b	2.30d	4.75c

† Means separation in each column by DNMRT, p = 0.05%

With 8 mg L^{-1} BA, a high number of shoots per explant and a significant reduction of plant height were obtained. The effect of BA on the number of leaf was significant. With an increase in concentration of BA the number of leaf decreased. Increasing the agar concentration up to 5.6 g L^{-1} significantly decreased shoot length. In an investigation (Pati *et al*., 2006), shoots with a better quality in terms of shoot length and thickness were achieved in a liquid medium compared to a gelled medium. Agar concentration did not significantly affect the number of leaves (Table 1). The influence of genotype on micropropagation of nine rose cultivars with proliferation rate of 1.85-2.88 plantlets per explant on medium supplemented with 3.0 mg L^{-1} of BA and 0.5 mg L^{-1} NAA has been observed by Carelli and Echeverrigaray (2002). The present data showed higher shoot number per explant and a non-significant plant height reduction in a higher BA concentration and lower agar concentration. Present findings indicated that to optimize a proliferation medium different concentration of BA and agar should be evaluated, based on genotype. The high shoot number with a suitable shoot length (3.83 and 3.27 mm, respectively) was observed at low concentration of agar (4.4 g L^{-1}) and high concentration of BA (8 mg L^{-1}) (Table 1).

Rooting and Acclimatization

With increasing the concentration of IAA, rooting increased up to 90% whereas the effect of salt concentration was not significant on rooting (data are not shown). Results revealed that effect of IAA on the root number was significant at 1% level of probability and the number of roots increased with an increase in the concentration of hormone. Mineral salt effect and its interaction with hormone were not significant on rooting. In other studies the rate of rooting increased in low concentration of mineral salts (Arnold *et al*., 1995; Siftar, 1996) or it was adequate for root inducing (Khosh-Khui and Sink, 1982b). It has been shown that rooting response of rose cultivars to auxin or medium salt concentration is complex and no clear relationship has been emerged (Arnold *et al*., 1995). Thus interaction between auxin, salt and plant genotype should be studied to determine a suitable nutrient rooting medium. In this study, however, the effect of mineral salt was not significant but the best result was obtained when a low concentration of MS salt was used. The highest root number was obtained in 6 mg L^{-1} IAA and ¼ MS salt (Table 2).

Effect of IAA and salt concentration was significant on root length. The longest root was achieved at ¼ MS salt concentration which is in accordance with the result of Hyndman *et al*. (1982).

After rooting, the plantlets were transferred to 3 soil mixtures. Among soil mixtures, the combination of sand + peat moss showed the highest root number (3.38) and plantlet height (16.1 cm) and the mixture of perlite + vermiculite showed the highest root length (11.3 cm) (Table 3). Jabbarzadeh and Khosh-Khui (2005) acclimatized Damask rose plantlets in a soil mixture consisting of peat moss and sand 1:1 (v/v) and successfully transferred to the greenhouse after 3 weeks.

The plantlets were kept under a plastic cover in a greenhouse at a mean temperature of 25±2°C and 60-70% relative humidity. The cover was gradually removed to acclimatize plantlets to *ex vitro* conditions (Fig. 1C). The plants were grown in the greenhouse successfully (Fig. 1D).

Table 2: Effects of different concentrations of MS salt and IAA on *in vitro* rooting of microshoots of rose cultivar 'Rafaela'

MS salt concentration	IAA	Root No.	Root length (mm)
1/4	0.0	2.88c†	12.95a
	1.5	7.96b	9.19c
	3.0	9.7ab	9.63b
	6.0	11.41a	9.6bc
½	0.0	3.77c	9.85b
	1.5	7.92b	8.92c
	3.0	9.05b	6.47e
	6.0	9.50ab	6.58de
3/4	0.0	4.13c	11.47ab
	1.5	8.77b	7.45d
	3.0	8.69b	8.48cd
	6.0	9.17b	6.94d

† Means separation in each column by DNMRT, p = 0.05%

Table 3: Effects of different soil mixtures on root number, root length and plantlet height 4 weeks after culture of rose cv. 'Rafaela'

	Rate of increase		
Soil mixture	Root No.	Root length (mm)	Plantlet height (cm)
Sand + peat moss	3.4a†	8.3b	16.1a
Peat moss	2.0b	3.2c	11.0b
Perlite + vermiculite	2.5b	11.3a	12.0b

† Each mean is the average of 50 plantlets. Means separation in each column by DNMRT, p = 0.05%

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