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Micropropagation and medium-term conservation of *Rosa pulverulenta*

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ABSTRACT. In Iran, a large number of *Rosa* species have been exposed to extinction and therefore preservation techniques are necessary to safeguard their future. In the present investigation, the objectives were to optimize the micropropagation and medium-term conservation of one of these wild species, *Rosa pulverulenta*. At proliferation stage, the maximum number of new leaves (9.6) were produced on the medium containing 4 μM BAP + 0 μM GA_3, whereas the maximum number of axillary shoots (4.1) were observed in the medium containing 4 μM BAP + 3 μM GA_3. The results for rooting experiments suggested that the highest increase in stem height (49.5 mm) at the acclimatization stage was observed in plantlets treated with 1 μM IBA + 0.5 μM NAA during the rooting stage. Comparing the refrigerator and phytotron conditions for medium-term conservation of *in vitro* plantlets indicated that although the refrigerator conditions resulted in lower growth rate compared with the phytotron, the survival rate in refrigerator (100%) was significantly higher than phytotron (87.5%). Furthermore, the growth rate of the plantlets from the refrigerator was accelerated during the recovery period and verged on to the ones stored in the phytotron.

Keywords: *in vitro* conservation, micropropagation, proliferation, recovery, rooting, *Rosa pulverulenta.*

RESUMO. Micropropagação e conservação durante médio prazo de *Rosa pulverulenta*. No Irã, um número grande de espécies de Rosa são expostas à extinção. Por causa disto, as técnicas de preservação são necessárias para garantir o futuro destas mesmas espécies. Nesta investigação, os objetivos foram aperfeiçoar a micropropagação e preservar durante médio prazo uma destas espécies, *Rosa pulverulenta*. No estádio de proliferação, o número máximo de novas folhas (9,60) foi produzido no meio contendo 4 μM BAP + 0 μM GA3, mas o número máximo de gemas axilares foi observado no meio com 4 μM BAP + 3 μM GA3. Os resultados obtidos permitiram inferir que o maior aumento na altura de haste (49,5 mm) durante o estádio de climatização foi observado nos explantes tratados com 1 μM IBA + 0,5 μM NAA durante o enraizamento. Observa-se que embora a geladeira tenha proporcionado uma menor taxa de crescimento, a sobrevivência em médio prazo foi maior (100,00%) do que no phytotron (87,50%). Além disto, a taxa de crescimento dos explantes que foram mantidos na geladeira foi acelerada durante o periodo de recuperação e ficaram próximas daquelas mantidas no phytotron.

Palavras-chaves: preservação *in vitro*, micropropagação, proliferação, recuperação, enraizamento, *Rosa pulverulenta*.

Introduction

Micropropagation has five major advantages compared to the conventional methods of plant propagation: (i) it is an invaluable aid in the multiplication of elite clones of intractable/ recalcitrant species; (ii) it is important in terms of multiplying plants throughout the year, with control over most facets of production; (iii) it is possible to generate pathogen-free plants even from explants of infected mother plants; (iv) plant materials such as male sterile, fertility maintainer and restorer lines can be cloned; and (v) it enables the production of a large number of plants in a short time from a selected number of genotypes (RANI; RAINA, 2000).

Micropropagated explants require less space and labor inputs for the maintenance of germplasm collections (RAJASEKHARAN, 2006). The *in vitro* conservation methods are considered particularly interesting for those horticultural species that are propagated by vegetative methods as well as those with recalcitrant seeds (SHIKHAMANY, 2006).

A variety of approaches have been used separately or in combination to reduce the growth rate of *in vitro* plant tissues. Probably, the most successful strategies have involved temperature reductions, but the

responses vary significantly between and within species. Slow growth is used as a short-term to medium-term conservation in many laboratories, including the Centro Internacional de la Papa (CIP) and Centro Internacional de Agricultura Tropical (CIAT) (ENGELMANN, 1991).

More than 200 species are present in the genus *Rosa* (WISSEMANN, 2003) from which 14 wild species are present in Iran. *Rosa pulverulenta* is a small bush (50 to 100 cm) with small pink (and rarely white) solitary flowers (10-25 mm in diameter) or clusters with 2-4 flowers. *R. pulverulenta* usually grows in Asia, south east Europe including west Syria, Caucasus, Azerbaijan, Armenia, Iran and Afghanistan (ERCISLI, 2005). Traditionally, most roses are heterozygous and do not breed true to type. Therefore, they are propagated by vegetative methods. Since most rose species are difficult to root, conventional propagating methods are very slow, time consuming, and tiring. Tissue culture on the other hand is becoming increasingly popular as an alternative to the conventional plant propagation methods (ROBERTS; SCHUM, 2003).

In Iran, a large number of *Rosa* species have been exposed to extinction and therefore preservation techniques are necessary to safeguard their future. The present investigation is the first report on *in vitro* propagation and medium-term conservation of one of these species, *R. pulverulenta*.

Material and methods

Plant material and culture conditions

Nodal segments (1-1.5 cm) of *R. pulverulenta* were collected from the *Rose* Germplasm Collection at the Agricultural Biotechnology Research Institute of Iran (ABRII). They were sterilized (KHOSRAVI et al., 2007) and transferred to induction medium containing full strength VS (VAN der SALM, 1994) mineral salts and vitamins, 2.7 g L^{-1} calcium gluconate and 2 μM 6-benzylaminopurine (BAP). The pathogen-free explants were then used in the proliferation and rooting stages of micropropagation.

The pH of all media was adjusted to 5.8 using 1.0 N potassium hydroxide (KOH) or 1.0 N hydrochloric acid (HCl), before adding 7 g L^{-1} plant agar. Media were autoclaved for 15 min. at 121°C and 1.2 kgf cm^{-1} pressure. All the *in vitro* cultures were placed under high pressure metal halide lamps (PPFD 60 μmol m^{-2} s^{-1} at the plant surface) on a 16/8 hour light/dark cycle in a culture room maintained at 20 ± 2°C.

In vitro propagation stages

Proliferation stage: Proliferation media contained full strength VS medium, 2.7 g L^{-1} calcium gluconate with various concentrations of BAP (0, 2, 4, 8 and 16 μM) in combination with giberellic acid (0 and 3 μM GA_3).

Elongation stage*: In vitro*, shoots were cultured on shoot elongation medium containing full strength VS mineral salt and vitamins, devoid of hormones, for 21 days before induction to rooting stage.

Rooting stage: Different levels of 3-Indolebutyric acid (IBA) (0, 0.25, 0.5 and 1 μM) in combination with 1-Naphtalene acetic acid (NAA) (0, 0.5 μM**)** were used in liquid media containing half strength VS salts, full amounts of vitamins, half levels of sucrose and 2.7 g L^{-1} calcium gluconate. The shoots were supported by the cellulose plugs called sorbarods (sorbarod, Ilacon, UK).

Acclimatization stage: Plantlets were acclimatized according to our previous report (KHOSRAVI et al., 2007).

In vitro conservation and recovery of shoots

Preservation of *in vitro* shoots encompassing 3 leaves was carried out in two conditions: refrigerator (4-6°C) in complete darkness and phytotron (20 ± 2°C) with 16/8 hour light/dark cycle (as control). All the shoots were cultured in the optimized proliferation medium (containing 4 µM of BAP and 3 µM of GA_3). The survived shoots were also transferred to optimized proliferation medium during the recovery phase.

Experimental design and statistical analysis

The shoot proliferation experiment was performed in a factorial based completely random design with 2 observations and 5 replications. Number of new leaves, ratio of green leaves to total number of leaves, plant height and number of axillary shoots were recorded after four weeks.

Rooting experiment, *in vitro* conservation and recovery of shoots were carried out in a factorial based completely random design with 3 observations and 5 replications. In the rooting stage, the percentage of rooting, number of roots per plantlet and total root length per plantlet were recorded after four weeks. In the acclimatization stage, the percentage of survival and stem height were compared in plants obtained from different rooting treatments after two weeks. Morphological parameters such as percentage of survival, number of new leaves, plant height, number of axillary shoots, and ratio of green leaves to total number of leaves were recorded after six months and four weeks for the conservation experiments and recovery phase, respectively. Analysis of variance was performed and comparisons of means were conducted using Duncan's Multiple Range test. All analyses were regarded as significant if p value was less than 0.05.

Results and discussion

Shoot proliferation

Maximum number of new leaves (9.6) was produced on the medium containing 4 μM BAP + 0 μM GA_3 (Figure 1A), whereas the ratios of green leaves to total number of leaves were not significantly different between most of the treatments (Figure 1B). The maximum number of axillary shoots (4.1) was observed in the medium containing 4 μM BAP + 3 μM GA_3 (Figure 1C) and the highest increase in plant height (0.71 cm) was observed in the medium containing 2 μM BAP + 3 μM GA_3 (Figure 1D).

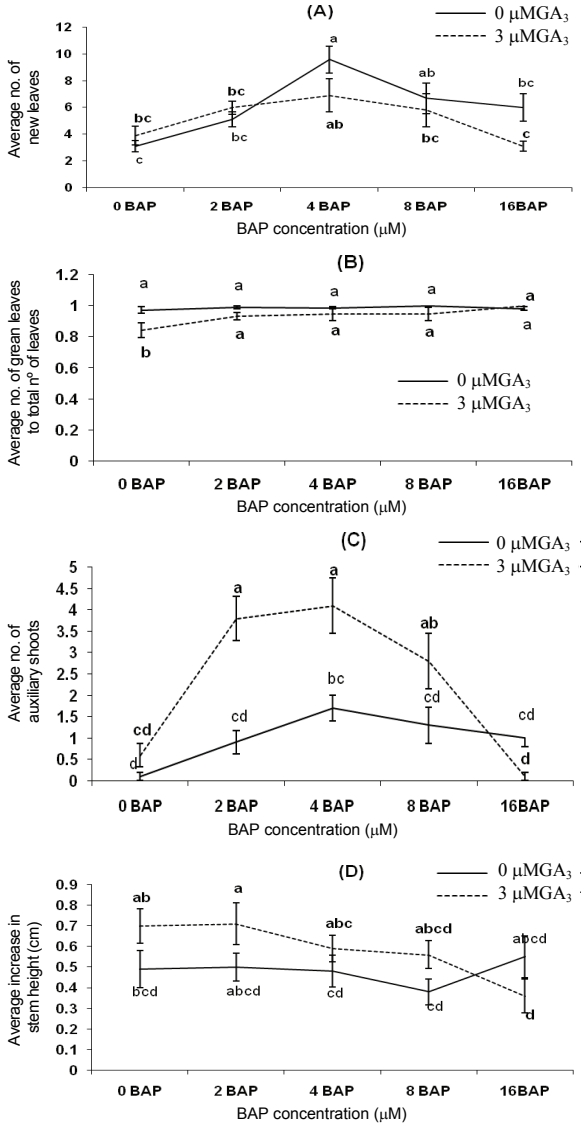

Figure 1. Comparing different treatments at the proliferation stage of *R. pulverulenta*, showing; A) Average number of new leaves B) Average ratio of green leaves to number of total leaves, C) Average number of axillary shoots D) Average increase in stem height (cm).
Different letters show significant differences according to Duncan's Multiple Range test ($p < 0.05$).

The results of the present study demonstrated that inclusion of 3μM GA_3 to the culture media significantly increased the number of axillary shoots and stem height in all of the BAP concentrations. Kim et al. (2003) indicated that *in vitro* shoot proliferation and multiplication are largely based on media formulations containing cytokinins as major plant growth regenerators, although low concentrations of auxins or GA_3 are also essential. The results showed that in some of the parameters the differences between the treatments were not significant, however, the maximum number of axillary shoots were significantly higher in the medium containing 4 µM of BAP and 3 µM of GA_3. As the concentration of BAP was raised to more than 4 µM, a reduced growth rate was noted (Figure 2A) which is in accordance with our previous report on *in vitro* propagation of *Rosa hybrida* cv. Iceberg where high concentrations of BAP resulted in reduced rates of growth (KHOSRAVI et al., 2007).

Root initiation and acclimatization

The Table 1 illustrates the response of *in vitro* shoots treated with media containing different concentrations of IBA and NAA. Although there was no significant difference among all the treatments in the percentage of rooting and even in the hormone-free medium, 73.3% of seedlings produced roots.

The highest percentage (93.3%) of rooting was obtained on the medium containing 0.25 μM IBA + 0.5 μM NAA. The longest root length per plantlet (64.7 mm) and the highest number of roots per plantlet (5) were recorded in the medium containing 1 μM IBA+ 0.5 μM NAA (Table 1). At acclimatization stage most of the plantlets from different rooting media had 100% survival rate, whereas the highest increase in stem height (49.5 mm) was observed in plantlets treated with 1 μM IBA + 0.5 μM NAA during the rooting stage (Table 2). According to Roberts and Schum (2003), roses are usually rooted in media containing different levels of salts and vitamins, often reduced to 1/4 or 1/2 strength, containing NAA, indole acetic acid (IAA), or both.

The present investigation recommended a combination of IBA (1 μM) and NAA (0.5 μM) for *in vitro* rooting of *R. pulverulenta*. This is in agreement with Roy et al. (2004) who also found that a combination of auxins (1 mg L^{-1} IBA and 0.5 mg L^{-1} IAA) was needed for *in vitro* rooting of roses.

Table 1. Effects of auxin concentration on the percentage of rooting, average number of root per plantlet and average root length per plantlet (mm) at the rooting stage (after four weeks) of *R. pulverulenta*.

Treatments	Rooting (%)	Average root length per plantlet (mm)	Average number of root per plantlet
0 μM IBA + 0 μM NAA	73.3^{a}	40.2^{abc}	2.5^{abc}
0 μM IBA + 0.5 μM NAA	73.3^{a}	27.0^{bc}	1.5^{c}
0.25 μM IBA + 0 μM NAA	86.7^{a}	29.0^{bc}	2.2^{bc}
0.25 μM IBA + 0.5 μM NAA	93.3^{a}	34.0^{abc}	4.2^{ab}
0.5 μM IBA + 0 μM NAA	73.3^{a}	60.0^{ab}	4.2^{ab}
0.5 μM IBA + 0.5 μM NAA	66.7^{a}	49.0^{abc}	3.0^{abc}
1 μM IBA + 0 μM NAA	80.0^{a}	23.5^{c}	2.8^{abc}
1 μM IBA + 0.5 μM NAA	86.7^{a}	64.8^{a}	5.0^{a}

Different letters show significant differences by the Duncan's Multiple Range test ($p < 0.05$).

Table 2. Effects of auxin concentration on the percentage of survival and average increase in stem height at acclimatization stage in *R. pulverulenta* after two weeks.

Treatments	Survival rate (%)	Average increases in stem height (mm)
0 μM IBA + 0 μM NAA	100.00	28.00
0 μM IBA + 0.5 μM NAA	100.00	45.63
0.25 μM IBA + 0 μM NAA	80.00	39.60
0.25 μM IBA + 0.5 μM NAA	50.00	49.50
0.5 μM IBA + 0 μM NAA	71.34	31.67
0.5 μM IBA + 0.5 μM NAA	100.00	36.67
1 μM IBA + 0 μM NAA	100.00	45.63
1 μM IBA + 0.5 μM NAA	85.50	41.60

In vitro conservation and recovery of shoots

The stored plantlets had higher survival rates in the refrigerator but had considerable higher growth rate in the phytotron. Percentages of survived seedlings in phytotron and refrigerator were 87.5 and 100%, respectively. Average increase in stem height, average number of new leaves and average number of axillary shoots of the plantlets stored in phytotron and refrigerator were 19.5, 42.2 and 3.53 mm and 2.1, 2.47 and 0.87 mm, respectively (Figure 2A and B). Comparing the ratio of green leaves to total number of leaves between the plantlets maintained in the phytotron and refrigerator indicated that there was no significant difference between them (Figure 2B). At the recovery stage, the growth rate of the plantlets stored in the refrigerator verged on to the ones that were stored in the phytotron. There was not a significant difference in average number of new leaves, ratio of green leaves to total number of leaves and average number of axillary shoots between the two storage conditions (Figure 2C and D). However, during the recovery period, average increase in stem height was significantly higher in the plantlets maintained in the refrigerator (4.8 mm) compared to the ones stored in the phytotron (1.1 mm) (Figure 2C). These observations are in agreement with Williams et al. (1997) who investigated the storage of *in vitro* shoot cultures of eight clones or cultivars of *Camellia japonica* L. and *Camellia reticulate* Lindley at 2-4°C for up to 12 months. They obtained 100% survival frequencies during the first or second sub-cultures after cold storage of seven in the eight clones assayed.

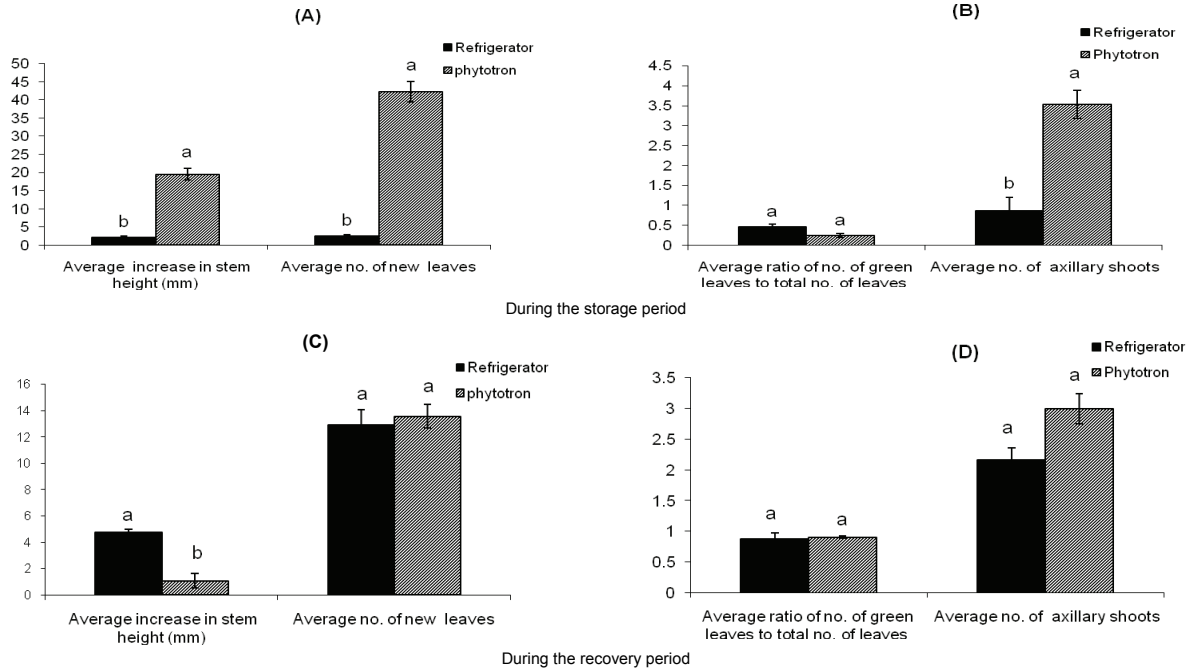

Figure 2. Comparing plantlets of *R. pulverulenta* conserved in refrigerator and phytotron, showing: A) average increase in stem height (mm) and average number of leaves during the storage period, B) average ratio of green leaves to total number of leaves and average number of axillary shoots during the storage period, C) average increase in stem height (mm) and average number of leaves during the recovery period, D) average ratio of green leaves to total number of leaves and average number of axillary shoots during the recovery period.
Different letters show significant differences according to Duncan's Multiple Range test ($p < 0.05$).

A combination of cold treatment and complete darkness slows the biological processes and hence the growth rate of plants. In the present investigation refrigerator condition resulted in lower growth rate of the plantlets, but the survival rate of the plantlets was significantly higher than those plantlets stored in the phytotron. Moreover, the growth rate of the plantlets from the refrigerator was accelerated during the recovery period. Therefore, storage in the refrigerator is considered an effective method for medium-term conservation of *R. pulverulenta*. Holobiuc et al. (2009) suggested that *in vitro* preserved material could be used as *ex situ* conservation source, and the effective propagation and medium-term conservation methods developed in the present study could be used as the basis for development of long-term conservation of *R. pulverulenta*.

Conclusion

In conclusion, the present investigation recommended a practicable, *in vitro* propagation and medium-term conservation protocol for *R. pulverulenta*. At the proliferation stage, maximum number of axillary shoots was achieved in the medium containing 4 μM of BAP and 3 μM of GA_3. At the rooting stage, a combination of IBA (1 μM) and NAA (0.5 μM) was eefective. The plantlets stored in the refrigerated had a low growth rate, but at the recovery stage, their growth rate verged on to the ones that were stored in the phytotron, therefore, storage in the refrigerator was considered an effective method for medium-term conservation and possibly for development of long-term conservation of *R. pulverulenta*.

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