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In Vitro Propagation of Damask Rose Using the Temporary Immersion System

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Abstract
For centuries, Damask rose has been one of the most popular species of the rose family. Damask rose can be propagated using vegetative methods such as micropropagation. In this research, the use of temporary immersion system was reported for the first time for Damask rose micropropagation. For this reason, a traditional temporary immersion system was designed to facilitate its micropropagation. To optimize this system, the effects of the BAP concentration, immersion time, explant size, number of explants per temporary immersion unit were investigated in the independent experiments using completely randomized design. Temporary immersion system was also compared with the solid and liquid media. The highest shoot number per explant was obtained from a temporary immersion system using an immersion time of 3 min every 150 min (11.8 shoots), a modified MS medium with 6 mg l^{-1} BAP (12.33 shoots) and explants with shoot length of 4 cm (12.45 shoots). Results indicated that the temporary immersion system was significantly better than solid and liquid media for the shoot number per explant (10.66, 2.56 and 5.66, respectively).

Keywords: BAP; Explant; Immersion time; Micropropagation; *Rosa damascena* Mill

Introduction
For centuries, rose has been regarded as the most popular ornamental plant in the world. Among the numerous species of the *Rosaceae* family, *Rosa damascena* Mill., known as Damask rose, is considered as one of the oldest and most valuable varieties. Its aroma and essential oils have therapeutic, sedative, antidepressant, antispasmodic, antioxidizing (Achuthan *et al.* 2003), antiseptic, antibacterial, antimicrobial (Basim and Basim 2003; Ozkan *et al.* 2004) and anti-HIV effects (Mahmood *et al.* 1996). In addition to having astringent agents and playing a role in altering blood cholesterol, it accounts for diverse world wide applications in the manufacture of various products.

Cutting, layering and grafting (Horn 1992) are the common vegetative methods of propagating *R. damascena* Mill. In some regions, the use of root suckers is the traditional and the most common rose propagation method. However, since it depends on season, sucker production in Damask rose has very low frequency (Mahmoudi Noodezh *et al.* 2012). Moreover, the methods of clonal propagation can't be fully relied on to attain healthy disease-free plants, thus resulting in slow rates of multiplication and subsequent reduction of production. Hence, the first widely accepted method of propagation of this plant in biotechnology was micropropagation as a popular large-scale of clonal propagation, through which Damask rose could be propagated to produce a

large number of plants within a small physical space and thus save time (Skirvin *et al.* 1990; Mahmoudi Noodezh *et al.* 2012) besides ensuring a disease-free plant production. Different features of tissue culture propagation systems for *R. damascena* have been described by several researchers. Supplementing the media with GA_3 (Kumar *et al.* 2000), 2.5-3.0 mg l^{-1} BA in combination with low rate of IBA (Jabbarzadeh and Khosh-Khui 2005) and 2 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and 100 mg l^{-1} phloroglucinol (Salekjalali, 2012) could improve *in vitro* multiplication of Damask rose. In another research the use of a liquid modified MS medium (with eliminated Cl^- and reduced NH_4^+ ions) caused the best growth of newly proliferated shoots and no aging was occurred (Nikbakht *et al.* 2005).

Notably, the development of the area under cultivation of Damask rose in some regions is necessary to produce rosewater and essential rose oils, which serve as the highly valuable commercial resource (Mahmoudi Noodezh *et al.* 2012). Since new large fields are needed annually to produce important crop species with high costs, conventional micropropagation has been limited commercially. To reduce the costs of micropropagation, automation via a bioreactor application has been proven to be an effective method (Aitken-Christie *et al.* 1995; Levin *et al.* 1998). Thus, the use of temporary immersion bioreactors (TIBs), which generally reduce the problems of hyperhydricity, poor quality of propagules and transplantation on a solid medium in the elongation and/or rooting stage usually confronted in the permanent liquid cultures, is simple and cost-effective. As a result, several commercial micropropagation laboratories have started the employment of the aforementioned systems for their productions. Furthermore, a superior mass balance, which resulted based on the use of TIBs, was associated with a higher proliferation rate, improved labor efficiency and reduced cost compared to the conventional micropropagation conducted on a semisolid medium (Teisson and Alvard 1995; Escalona *et al.* 1999; Etienne *et al.* 2006). In the TIBs, cultures are temporarily immersed in the medium at specified intervals with a preset duration. A typical design involves the utilization of two plastic or glass vessels; one holds the liquid medium and the other the explants (Adelberg and Simpson 2002). Several studies have been carried out to investigate the use of automated temporary immersion systems to study the effects of environmental conditions, such as nutrient supply and internal atmosphere composition in the culture vessel, on the physiology of some recipient species (Martre *et al.* 2001; Escalona *et al.* 2003). The use of temporary immersion system has been reported in other plants such as hybrid chestnut (Vidal *et al.* 2015), teak (Quiala *et al.* 2012), pistachio (Akdemir *et al.* 2014) and peach palm (Steinmacher *et al.* 2011). The use of temporary immersion system in Damask rose micropropagation is yet to be studied. Therefore, in this research, a traditional temporary immersion system was designed and thereafter the effects of BAP concentration, immersion time, explant length and number of explant per temporary immersion unit were investigated on the shoot number per explant.

Materials and Methods

For the micropropagation of Damask rose, a temporary immersion system (T.I.S.), the BIT (Twin Flask System) type (Escalona 1999), was designed and used. The different parts of this system include silicone tubes, air filters, programmable timers, air compressors, solenoid valves and glass vessels. The system applied here included two glass containers (volume, 600 ml), one of which was used for the growing of *in vitro* plantlets while the other served as a liquid medium reservoir (150 ml). The silicone tubes are used as the channel for the transfer of compressed air between the compressor and the vessels and also the transfer of culture medium between the vessels. The airflow was sterilized when passing through the hydrophobic filters of 0.45 μm and the air pressured by an air compressor pushed the medium from one container to the other, so that the explants could be immersed and the shoots could be regenerated. Then, to withdraw the medium from the container of culture, the airflow was reversed. Meanwhile, the programmable timers controlled the immersion period frequency and length (Figure 1).

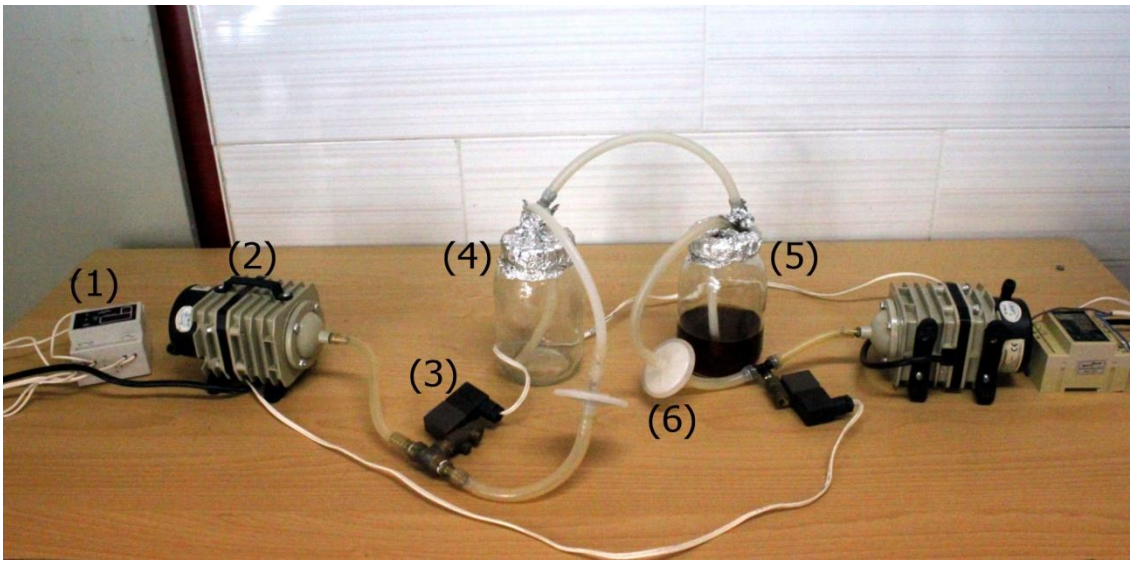

Figure 1. Temporary immersion system (1: programmable timer, 2: air compressor, 3: solenoid valve, 4: the first glass container for growing plant materials, 5: the second glass container was used as a liquid medium reservoir, 6: air filter)

The explants used in this study were prepared from a single Damask rose (cultivar Azaran) plant grown in a Damask rose garden. The height of the shrub was between 100 and 150 cm, and this shrub had 10 to 20 one-year stems, with each stem having approximately 20 to 25 axillary buds. The nodal explants (about 1.5 cm in length, with one axillary bud) from the mid-stem region of one-year stems (0.4–0.6 cm in diameter) were chosen as explants.

Initially, the leaves subtending each bud were cut at the base of the petiole, the plant materials (stems with 10 cm in length with a maximum of four buds) were then washed with liquid detergent (common dishwashing liquid) diluted in water and

were placed under running tap water for 30 min. The nodal sections were then initially treated with 5.25% sodium hypochlorite (w/v) for 10 min, followed by rinsing with sterile distilled water. Afterward, the buds were rinsed with 70% ethanol for 30 s, followed by rinsing in the sterile distilled water and a further disinfection was done in 5.25% sodium hypochlorite solution (w/v) with five drops of Tween-20 added for 30 min, and this solution was gently stirred with a magnetic stirrer. Finally, the explants were rinsed three times with sterile distilled water (Mahmoudi Noodezh *et al.* 2012). The base medium was the modified MS medium, A_{19} (amount of NH_4NO_3 and $CaCl_2$ increased 1.5 times, iron supplementation (FeEDDHA) increased about 20%) supplemented with 4 mg l^{-1} of BAP, 0.25 mg l^{-1} IAA, 50 mg l^{-1} ascorbic acid, 50 mg l^{-1} citric acid, 3% sucrose, 6 g l^{-1} agar-agar and 2 g l^{-1}phytagel) (Mahmoudi Noodezh *et al.* 2012). Glass bottles with autoclave-resistant plastic caps (5.5 cm in diameter, 8 cm in height and 250 ml in volume) containing 50 ml of modified MS medium (pH 5.8) were autoclaved for 20 min (121 °C and 1.2 bars). The cultures with three nodal explants per bottle were incubated at 24±1°C in a controlled growth chamber under a 16-h photoperiod with a light intensity of 3000 lux. After 15 days, for studying the effects of the BAP concentration (2, 4, 6 and 8 mg1^{-1}), immersion time (3 min every 90, 150 and 300 min) and the number of explants per temporary immersion unit (3, 4 and 5 explants), in three independent experiments, the cultivated explants together with their regenerated shoot (mean length of the shoots about 1.5-2 cm) were selected and transferred to the temporary immersion system. In another experiment, effect of the size of the regenerated shoot on each explant was investigated. Therefore, the explants with different shoot size (1, 2, 3 and 4 cm in height) were selected and transferred to the temporary immersion system. Also temporary immersion system was compared with the liquid and solid A_{19} media in the same glass bottles. Each experiment was performed as a completely randomized design layout with three replications (each replication consisted of three explants per temporary immersion unit). After 30 days the mean shoot number per explant was recorded. According to our previous research on Damask rose micropropagation (Mahmoudi Noodezh *et al.* 2012) ½ A_{19} medium supplemented with 2 mg l^{-1} of IBA was used for rooting some of the best regenerated shoots in the temporary immersion system (a separated rooting experiment has not been performed). Then the plantlets were transplanted to small plastic pots containing perlite and maintained in a controlled growth chamber at 25±1 °C and high Relative Humidity (RH) under a 16 hr photoperiod. RH was gradually reduced and after about 20 days the plants were transferred to large plastic pots containing perlite and cocopeat (1: 5 v/v) and transferred to a glass greenhouse at 27± 2 °C.

Preliminary statistical analyses such as normality test (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) were conducted. After analysis of variance, the means of treatments were compared using Duncan's multiple range procedure. All statistical analyses were carried out using SPSS version 14.

Results and Discussion

The results of analysis of variance indicated a significant difference between the different concentrations of BAP at 1% probability level of for the mean shoot number per explant. The mean comparisons (Figure 2) showed that the use of 6 mg l^{-1} BAP produced the highest mean shoot number per explant (12.33 shoots). There was no significant difference between 4, 6 and 8 mg l^{-1} BAP, but the use of 4 mg l^{-1} BAP was more suitable due to the production of shoots with better quality and less vitrified tissues. In the solid medium (control) containing these BAP concentrations, the shoots did not grow well enough and these concentrations apparently exhibited poisonous effects. In the temporary immersion system, a high concentration of BAP (6 mg l^{-1}) did not show poisonous effect due to the temporary contact between the liquid medium and plant material, while there was a permanent contact in the continuous liquid or solid media.

In the second experiment, analysis of variance showed a significant difference between the different immersion times at the 1% probability level for mean shoot number per explant. The comparison of means (Figure 3) indicated that the immersion-treatment of 3 min every 150 min produced the highest mean shoot number per explant (11.86 shoots), while the control (solid medium) had the lowest mean shoot number per explant (2.67 shoots). Every plant species owns a specific immersion time for *in vitro* growth and development, which is a key factor for the use of temporary immersion system in managing the uptake of nutrients and expression of hyperhydricity. A variety of immersion times have been considerably used by different researchers and this is probably due to the presence of a large variety of species, micropropagation processes and temporary immersion systems (Hvoslef-Eide and Preil 2005). Based on immersion time, efficient tuberization of potato and somatic embryo production in coffee and rubber would take a long (1 h every 6 h) and very short (1 min every 12 h) times, respectively (Etienne *et al.* 1997a, 1997 b), while for the propagation of grapevine shoots, very frequent immersions (30 s every 30 s) prove to be of a high efficiency (Harris and Mason 1983). Immersion frequencies were further reported to be important in the proliferation of service berry shoots. Additionally, hyperhydricity was seen to occur with immersions of 5 min every 30 min, but not 5 min every 60 min (Krueger *et al.* 1991).

The analysis of variance of the third experiment showed also a significant difference between the treatments for mean shoot number per explant. The mean comparison (Figure 4) showed that the use of explants with shoot length of 4 cm in the temporary immersion system produced the highest mean shoot number per explant (12.45 shoots) and explants with shoot length of 1 cm had the lowest mean shoot number per explant (8.33 shoots). The comparison of means showed that the explants with more length produced more shoots in the temporary immersion system. The explant length was directly correlated with the number of

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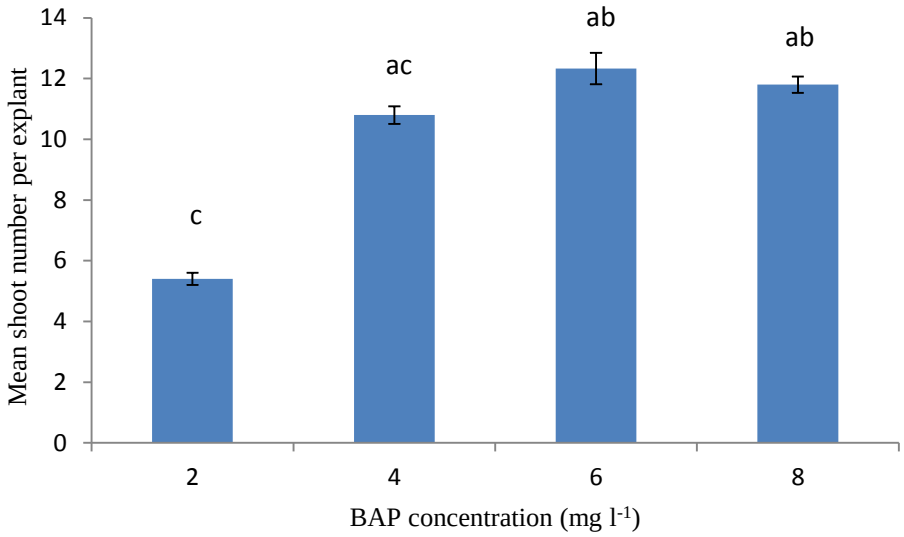

Figure 2. Mean comparison of BAP concentrations in Damask rose micropropagation using temporary immersion system in terms of the mean shoot number per explant

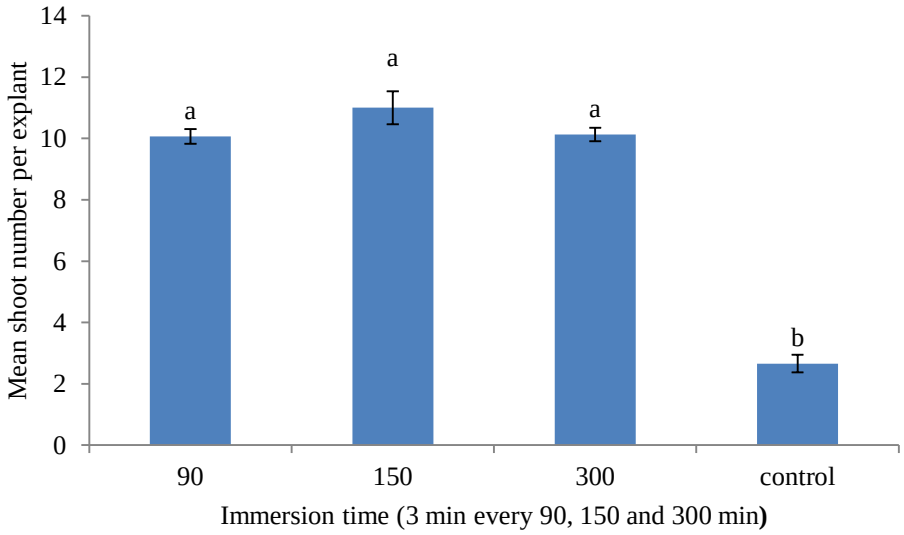

Figure 3. Mean comparison of the different immersion times for the mean shoot number per explant in Damask rose micropropagation using temporary immersion system

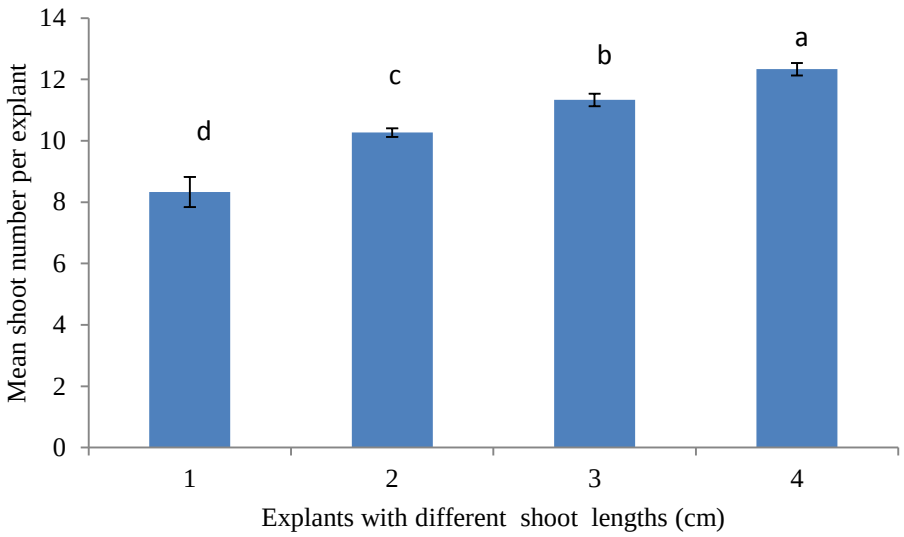

Figure 4. Mean comparison for the explants with different shoot lengths in terms of the mean shoot number per explant in Damask rose micropropagation using temporary immersion system

regenerated shoots. The longer and stronger explants had the higher number of lateral buds, as well as bigger lateral buds. In a study, it was shown that initial explants have a positive effect on growth and reproduction rates in cacao (Traore *et al.* 2003).

In the fourth experiment, there was no significant difference among different number of explants (3, 4 and 5 explants) per temporary immersion unit. This can be an important advantage for this propagation system, since more explant number will produce more regenerated shoots.

The analysis of variance for the comparison of the temporary immersion system with the solid and liquid media showed that there was a significant difference between the treatments. The immersion system (Figure 5) produced the highest mean shoot number per explant (10.66 shoots), while the solid medium had the lowest mean shoot number per explant (2.56 shoots).

In general, these results showed that the temporary immersion system is a suitable method for Damask rose micropropagation. In this method, the highest mean shoot number per explant was obtained using 6 mg l^{-1} BAP (12.33 shoots per explant), immersion time of 3 min in every 150 min (11.8 shoots per explant) and explants with shoot length of 4 cm (12.45 shoots per explant). Compared to agar-based cultures, plant micropropagation in a temporary immersion system provides several advantages, including better control of plant tissue-culture medium contact, aeration, medium circulation and culture scaling-up and optimal supply of nutrients and growth regulators. In conclusion, these results showed that the temporary immersion system is a potential method used in the micropropagation of Damask rose, which resulted in significant improvements of shoot regeneration and

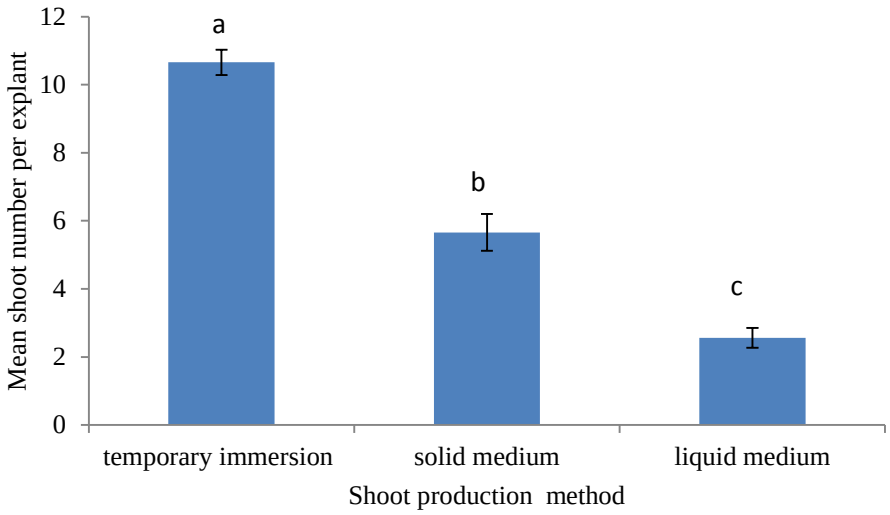

Figure 5. Mean comparison of the temporary immersion, solid and liquid media for the mean shoot number per explant in Damask rose micropropagation

Figure 6) a: *In vitro* shoot proliferation of Damask rose in the temporary immersion system, b: The shoots regenerated from one explant in the temporary immersion system

proliferation. The optimum micropropagation in the temporary immersion system will depend on a better understanding of plant responses to every specific microenvironment and on specific culture manipulation to control the morphogenesis of regenerated plantlets in this propagation method.

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