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Comparative study of *Rosa damascenes* Mill. and *R. Gallica* micro-propagation

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ABSTRACT: Roses are an important commercial cut flower with a wide range of varieties throughout the world. Due to its economic value, this study aimed to optimize the in vitro propagation protocol of three rose cultivars i.e. two (Kashan and Kazanlik) from *Rosa damascene* Mill. and one (Tuscany superb) from *R. gallica* by comparing the micro-propagation stages of the musing nodal explants. Using nano-silver in the medium with concentrations of 0, 25, 50 and 75 µmol in a completely randomized design. Result showed that the quality of Kashan cultivar explant increased while it had adverse effect on the other cultivars. The results obviously showed that liquid medium containing 1.5 mgl^{-1} 6-benzylaminopurine (BAP) affected proliferation depending on rose cultivar. Modified Murashige and Skoog (1962) (MS) medium fortified with 2.0 mg l^{-1} (BAP) and 0.2mgl^{-1} indole-3-butyric acid (IBA) gave optimal shoot proliferation. Micro shoots from BAP induced cultures could be rooted easily on indole-3-butyric acid (IBA) supplemented medium. Treatment with IBA (2 mgl^{-1}) was very effective for root induction. Effect of plant growth regulators on proliferation and rooting was also different depending on cultivar. The rooted plants were successfully transferred to pots after 15 d of hardening in a mist chamber. The present work highlights the significance of rose species and cultivars difference during micro propagation.

Key words: Damask rose, *R. gallica*, micro-propagation, in vitro rooting, and liquid medium

INTRODUCTION

High commercial value and extensive cultivation of roses make them one of the main important ornamental crops to propagate. Among the known rose species, just a few have been recognized as "scented roses". *Rosa damascene* Mill (Damask rose) is one of the oldest and most valuable varieties. This rose is also used worldwide for the manufacture of products with diverse applications such as aroma therapeutic, antidepressant, antiseptic, antispasmodic, astringent agent, sedative, blood cholesterol altering, antibacterial and antimicrobial (Basim and Basim 2003; Ozkan *et al.* 2004), antioxidizing (Achuthan *et al.* 2003), and anti-HIV effects (Mahmood *et al.* 1996). Furthermore, *R. damascene* is used in the production of perfume, cosmetic, and food industries including rosewater, jam, and dried flowers (Mahmoudi Noodezh *et al.* 2012).

Vegetative methods are utilized for propagation of roses using cuttings, layering, budding and grafting. Propagation of new cultivars and rootstocks is done by seeds (Horn, 1992). Root suckers are traditionally used for rose propagation in Iran but its multiplication rate is slow which decreases production and economic revenue.

Now, tissue culture technique is becoming an important method for massive production of stocks for this commercial plant species. It makes possible the production of a large numbers of plants within a small physical space and in a short period of time. Among roses, there is no report on in vitro propagation in *R. gallica* but information is available for *R. damascene* (Khosh-Khui and Sink, 1982; Ishioka and Tanimoto, 1990; Koronova and Michailova, 1994; Kumar *et al.*, 2001, Mahmoudi Noodezh *et al.* 2012).

We performed a parallel study on different stages of micro-propagation, such as the initiation of aseptic culture, shoot proliferation, rooting and plantlet establishment for *R. damascene* and *R. gallica* and compared their responses. The present work also highlights the significance of nano-silver (NS) and FeEDDHA for enhanced proliferation performance.

MATERIALS AND METHODS

A. Plant material and Surface sterilization of explants

The experiments were carried out in the Tissue culture Laboratory, Department of Horticultural Science, University of Tarbiat Modares University (TMU) Tehran, Iran, during the period from 2013 to 2014.

Plant materials were obtained from 3-year-old *R. damascene* vs. Kashan and Kazanlik and *R. gallica* cv. Tuscany superb vegetative rootstock grown in the green house with normal daylight and temperature range of 20-35°C during night/day. Nodal segments taken from actively shoots with length of 15-20 cm were used as the source of explants.

The explants were first washed with water containing of a mild commercial detergent by continuous shaking, followed by washing under running tap water for 30 min. Then, they were disinfected with 1mg l^{-1} benomyl powder, a broad spectrum fungicide for 20 min. Further washing was done under a laminar air flow, where explants were treated with 50% (v/v) sodium hypochlorite for 10 min, followed by rapid washing with sterilized distilled water and finally, rinsed with sterilized distilled water (at least 3 times). After sterilization, the nodal segments were then washed with 50 mg l^{-1} sterilized citric acid for 2-3 min. and inoculated on sterile media.

B. Culture media and conditions

The Murashige and Skoog (1962) (MS) medium containing 3% (w/v) sucrose and 0.8% (w/v) agar were used in the most of the experiments (except liquid culture in which medium was prepared without agar). The Ph of the medium was adjusted to 5.8 using 1 N NaOH or 1 N HCl.

The media were sterilized by autoclaving at 1.2 bars pressure and 121°C temperature for 20 min. All the cultures were maintained at 24±2°C under a 16-h photoperiod with a light intensity of 3000 lux.

C. Establishment stage

Culture medium. Sterilized single node explants were inoculated in McCarthy glasses containing 15 ml woody plant medium (WPM)(Lloyd and McCown, 1980) and MS media with 0, 25 and 50 mg l^{-1} benzyl amino purine (BAP) and 0 and 0.2 mg l^{-1} indol-3-butyric acid (IBA)distributed in McCarthy glasses were used for establishment of single nodes. After 17 days, leaf number, shoot length, shoot number and establishment percent were recorded.

At the same time and in another experiment, Sterilized single node explants were inoculated in McCarthy glasses containing 15 ml modified MS (mMS) medium (Table 1). This media were supplemented with various concentrations of NS solution (Nanocid Colloid L, 2000, Nanopars Ltd, Tehran) (0, 25, 50 and75mg l^{-1}). Quality of plantlets was scored 1-5 based on growth parameters such as vitrification, STN (shoot tip necrosis), yellowing, leaf area, and leaf quality and explants which exhibit normal growth were given the highest score.

Table 1: Modified concentrations of MS macronutrients (mMS medium).

Macro elements	Concentration (mg l^{-1}l)
NH_4NO_3	1200
KNO_3	1900
$MgSO_4$	190
KH_2PO_4	170
$Ca(NO_3)_2$	600

D. Shoot proliferation stage

Culture medium composition. In order to evaluate nutrients of different culture media on shoot multiplication, the effect of various ratios of NH_4NO_3: KNO_3 were evaluated (Table 2). 45 days after inoculation chlorosisrate, shoot length, number of shoots and number of nodes were recorded. The effects of MS and mMS media containing 1.5 mg l^{-1} BAP were also compared. Number of shoots and the amount of chlorophyll of leaves (Arnon, 1949) were evaluated after 4 weeks.

Table 2. Modified concentrations of MS medium.

Medium	NH_4NO_3/ KNO_3mg l^{-1}	Concentration of another elements	
1	2475/1900	$MgSO_4$	190
		KH_2PO_4	170
		$CaNO_3$	600
2	1200/1900		
3	1980/2280	In all medium concentration the other elements similar basal MS medium.	
4	1450/1700		
5	1320/1520		
6	1000/1500		

Chlorophyll a and b were detected using the following formulas:

$$Cla = \frac{(19.3 \times A663 - 0.83 \times A645) \times V}{100 \times w}$$

$$Clb = \frac{(19.3 \times A645 - 3.6 \times A663) \times V}{100 \times w}$$

Where *V* is the volume of used solution, *W* is fresh weight (*g*) of the sample and *A* is light absorbtion at 663 and 645 nm wavelength.

mMS media containing 1.5 mg l^{-1} BAP or thidiazoroun (TDZ) were used for comparison of cytokinin type effect. Shoot and node number, shoot length and quality index were assessed after 4 weeks. Quality index was assessed by scoring 1 to 5, from weak to excellent, respectively.

mMS agar gelled and liquid media containing 1 and 2 mg l^{-1} BAP were used for proliferation, as well. Number of shoots, shoot length, number of leaves and nodes were recorded after 4 weeks.

E. Callus induction

Ex vitro leaves of Kashan and Kazanlik were used for callus induction study. Scarified leaves were initiated onto MS medium containing 1.5 and 2.5 mg l^{-1} 2, 4-D. Inoculated media were incubated in darkness for 2 weeks and then returned to the growth room. The weight of produced callus was measured after 2 months.

F. In vitro rooting and plantlet acclimatization

Proliferated shoots of the three genotypes were inoculated on half strength MS (1/2MS) and Linsmaier and Skoog (1965) (LS) media containing 1 and 2 mg l^{-1} IBA for *in vitro* rooting. Root number, length and rooting percent were recorded after 4 weeks. Micro shoots were transferred in the control medium (1/2 MS and LS) without any hormones not significant thus results didn't shows in this paper.

Rooted plantlets were subjected to hardening using 10 in 15 cm autoclaved jam jars containing jiffy peats soaked in liquid 1/2MS inorganic salts. The rooted plantlets were washed in sterile distilled water containing 0.1% Benomyl and then transferred individually to each container. Cultures were kept under controlled photoperiod and temperature condition as previously described for nodal cultures. During the 3rd week, the caps were loosened and gradually removed to reduce the humidity. During this stage, the plantlets were irrigated regularly with one-fourth MS salts solution minus organics. Plants were then transferred to plastic pots filled with sand and soil in a glasshouse during the 6-8th week of hardening depending on the genotype.

G. Statistical analysis

Experiments were conducted with at least 5 replicates per treatment and each consisted of 5 to 6 propagules. Completely randomized design was used for all experiments. The data were analyzed statistically using SAS 9.2 (SAS Institute Inc., 2008). The significance of difference among means was carried out using Least Significant Difference (LSD) test at $P< 0.05$.

RESULTS AND DISCUSSION

A. Sterilization and establishment of nodal explants

Effect of Sodium hypochlorite. Using different concentrations and times of NaHClO exposure for sterilization of explants, the highest initiation percent of new shot growth was obtained in 2.5% for 5 min. (Fig. 1) and, the lowest contamination percent was detected in 2.5% for 5 and 7.5 min (Table 3). In the present study, we found that at concentrations higher than 2.5% NaHClO and for times longer than 5 min., nodal explants showed apical necrosis and so explants' growth was interrupted. As well, the percentage of contamination increased in exposure times shorter than 5 min. Hesabi (2011) used 5.25% NaHClO for 3 min for Damask rose explant sterilization. It has been detected that NaHClO destroys cell membranes and microorganisms' DNA (Jang *et al.*, 2008).

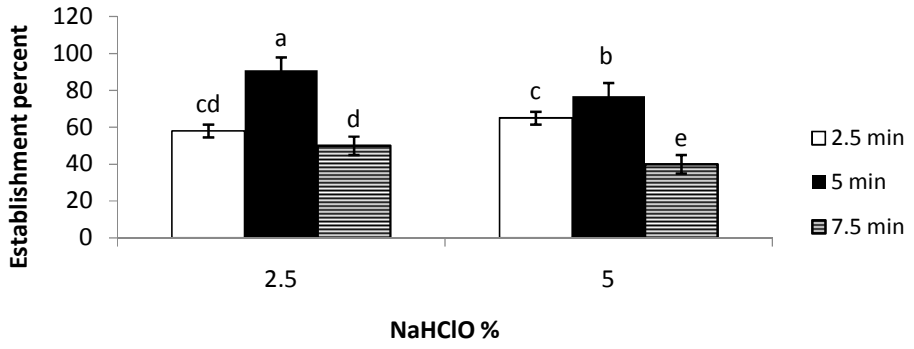

Fig. 1. Effect of sodium hypochlorite on *in vitro* establishment of Damask rose cultivars.

Table 3: Effect of NaHClO on contamination of nodal explants of Damask Rose.

Exposure to NaHClO (min.)	Contamination%
2.5	0.56±0.21a
5	0.37± 0.09 b
7.5	0.34± 0.66 b

Effect of plant growth regulators. Different cultivars showed various reactions to the IBA treatments. The highest establishment percent (%79) was achieved for Kashan with 0.2 mg l^{-1} IBA which was not significantly higher than Tuscany superb on 0.2 mg l^{-1} (73%) and 0 mg l^{-1} (74%). The lowest establishment percent was also detected in Kashan cultivar but on 0 mg l^{-1} IBA (control) (57%) with non significant difference with kazanlik on 0.2 mg l^{-1} IBA (62%) (Fig. 2). New shoot containing young shoot and few leaves induced after 2 weeks. Effect of nano-silver. NS used in the culture medium showed significant effects on initiation and establishment percent of nodal explants of three rose cultivars. 25 mg l^{-1} NS in Kashan cultivar showed the highest establishment percent with non significant difference to the control. None of the cultivars showed significant difference in establishment with NS treatments in culture medium except Tuscany superb which had significantly lower establishment percent when 75 mg l^{-1} NS was added to the culture medium. So, Tuscany superb was sensitive to high concentrations of NS (Fig. 3) such that its shoot length decreased significantly using NS (Fig. 4). The highest shoot length detected in Kashan on medium containing 25 mg l^{-1} NS was not significantly different from the control. Kazanlik was not affected by the NS in culture medium. Number of leaves produced by the studied cultivars were not affected by NS (Fig. 5). However, the quality index of Kashan established explants increased using NS in the medium but it decreased in Kazanlik with 75 mg l^{-1} NS. It did not have any effect on Tuscany quality index (Fig. 6). The Positive effect of NS on explant quality index could be because of its effect on absorbing ethylene produced by the *in vitro* explant.

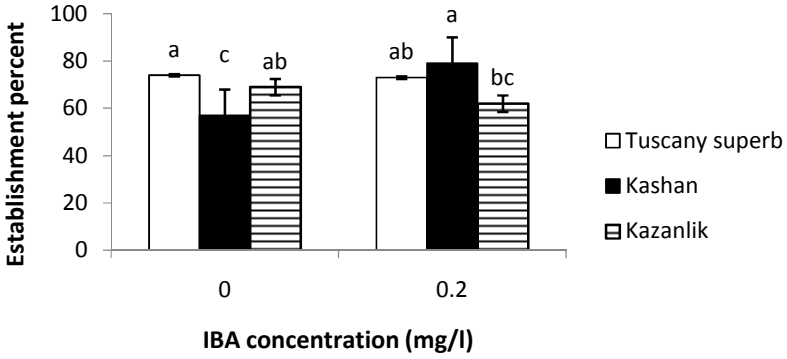

Fig. 2. Effect of IBA concentration on *in vitro* establishment of Damask rose cultivars.

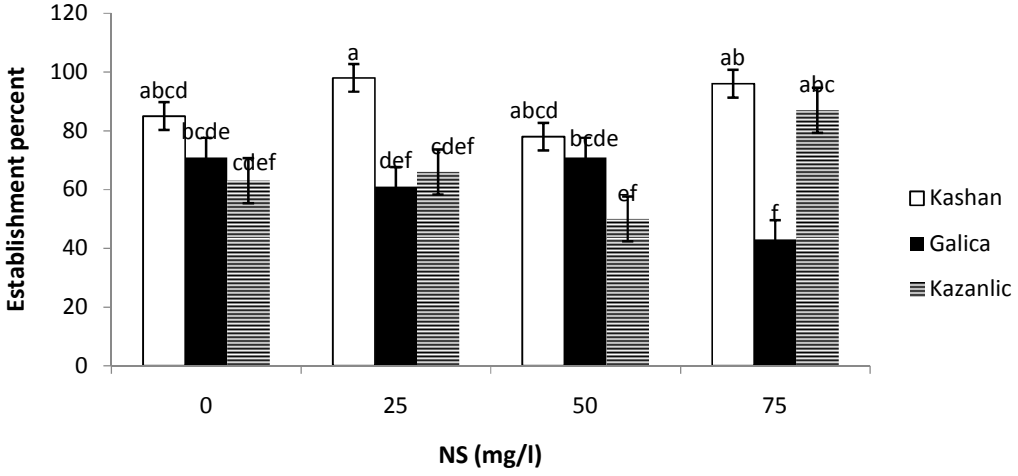

Fig. 3. Effect of Nano-silver concentrations used in culture medium on *in vitro* establishment of rose cultivars.

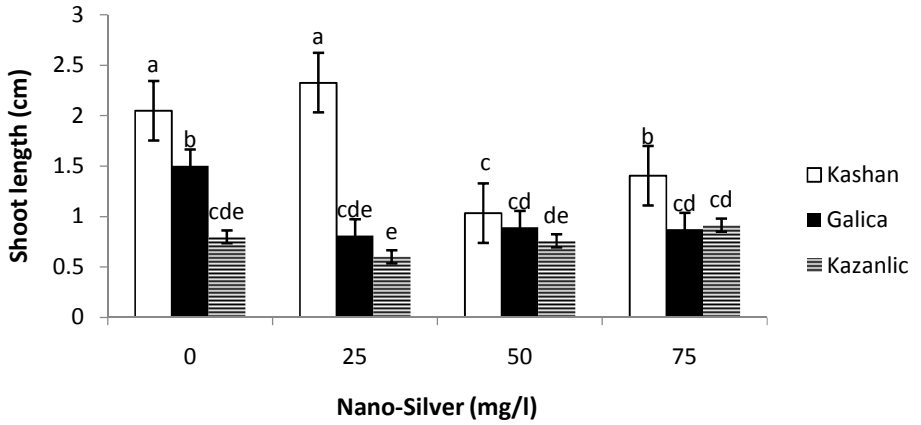

Fig. 4. Effect of Nano-silver concentrations in culture medium on shoot length of Damask rose cultivars.

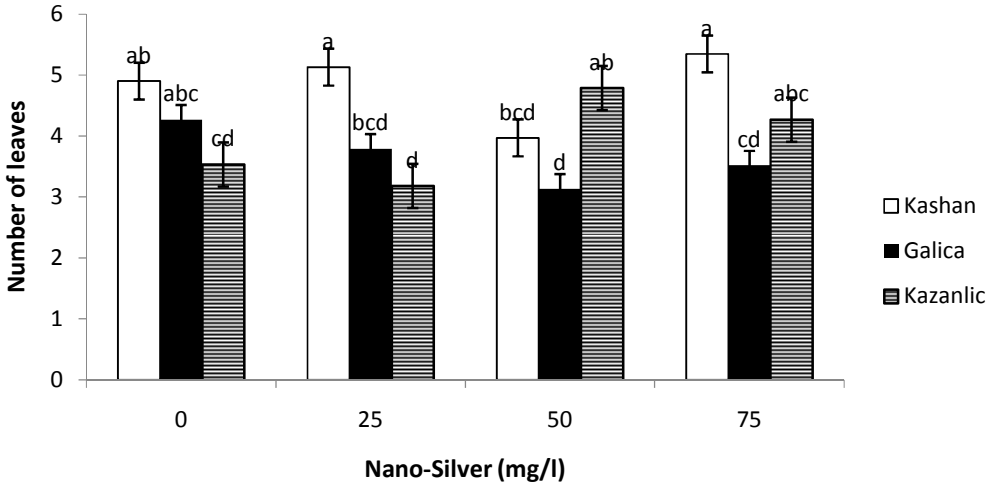

Fig. 5. Effect of Nano-silver applied*in vitro* culture medium on number of leaves of Damask rose cultivars.

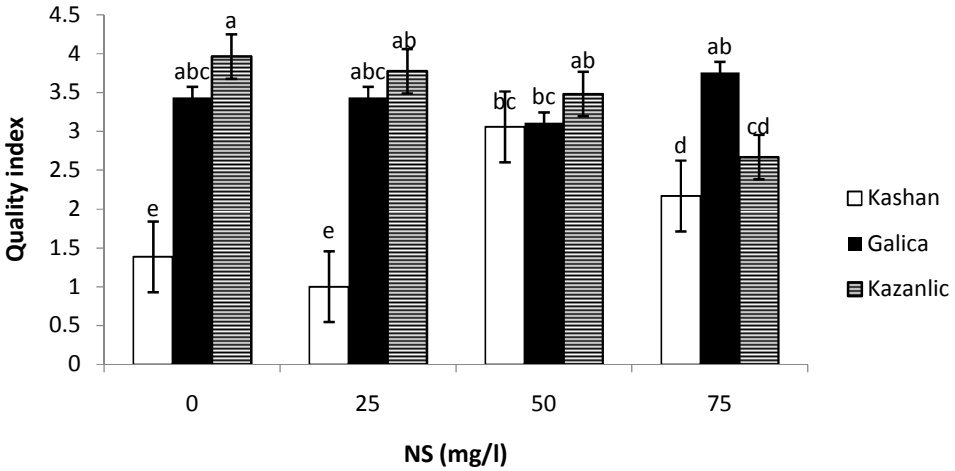

Fig. 6. Effect of Nano-silver concentrations in culture medium on quality index of *in vitro* established explant of Damask rose cultivars.

B. Shoot proliferation

Effect of culture media composition. Six media differing in the concentrations of NH_4NO_3/KNO_3 were tested in the proliferation phase (Table 1). The results, presented in Table 4, clearly show that treatment 4 resulted in the highest number of leaves (23.2) and shoot length (4.8 mm). It also resulted in a high number of shoots (1.1), number of nodes (1.5) and callus weight (1.6 mg) which were not significantly lower than the highest ones. But the quality index was highest in treatment 2 (0.6) (Table 4). The result show that the high level of chlorophylls was observed in mMS medium (Table 7).

Table 4: Effect of different culture medium composition on shoot proliferation.

Medium	Number of leaves	Shoot length (cm)	Number of shoots	Number of nodes	Callus weight (mg)	Quality index
1*	12.5±0.00 b**	3.5 ±0.01ab	1.2±0.11 a	1.21±0.02bc	1.43±0.025a	0.25± 0.01 b
2	16.0 ±0.02ab	3.8±005ab	1.2±0.45 a	1.11±0.52bc	1.55±0.62a	0.57±0.06 a
3	13.4±0.11ab	3.7 ±0.01ab	1.29 ±0.41 a	1.30±0.41bc	1.34±0.021a	0.34±0.12ab
4	23.1±0.01a	4.8±0.07 a	1.11± .33 a	1.51±0.06ab	1.55±0.6a	0.14± 0.05 b
5	4.0±0.04 c	1.8±0.02c	0.51 ±0.71b	0.97 ±0.33 c	1.07 ±0.014b	0.14±0.42 b
6	12.0±0.04b	3.4±0.03 b	1.15 ±0.01a	1.82 ±0.08a	1.53±0.35a	0.26± 0.01 b

*1, 2, 3, 4, 5 and 6: Media according to table 2; **same letters in a column are not significantly different

MS is the most common basal medium used for rose micro-propagation (Hasegawa, 1980). Specifically, modified MS and ½MS media have been used successfully in various studies on the *in vitro* propagation of Damask rose (Bordbar 2005). Some researchers have proposed that decreasing ammonium salts in Damask rose culture medium (particularly $[NH_4]_2SO_4$ and NH_4NO_3) may lead to improved micropropagation (Bressan *et al.* 1982; Curir *et al.* 1986; Valles and Boxus 1987a, b).

But recently, Mahmoudi Noodezh *et al.* (2012) showed that increasing ammonium nitrate resulted in increased growth and overall strength of the shoots, with production of dark green and healthy leaves. In contrast, we studied the effect of alterations in NH_4NO_3 along with KNO_3 or other macronutrients and it was concluded that not only increasing or decreasing NH_4NO_3 can affect the quality and quantity of proliferation but also it depends on alterations on other macronutrients like KNO_3. Comparative proliferation in liquid and agar gelled media. Liquid medium significantly increased the shoot length of the Kazanlik and Kashan cultivars but not Tuscany (Fig. 7). Agar gelled medium was effective usingKazanlikas the number of nodes was significantly more in solid medium (Fig. 8).

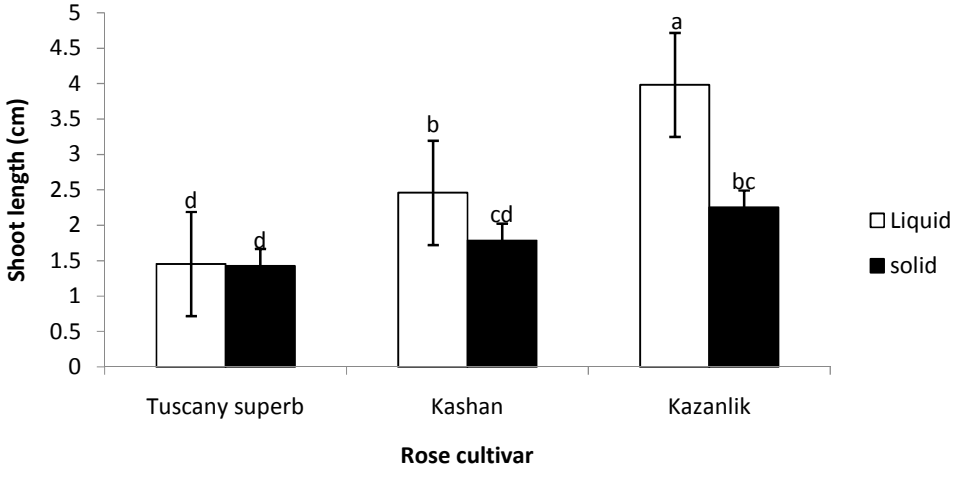

Fig. 7. Effect of medium type on *in vitro* shoot length of Damask rose cultivars.

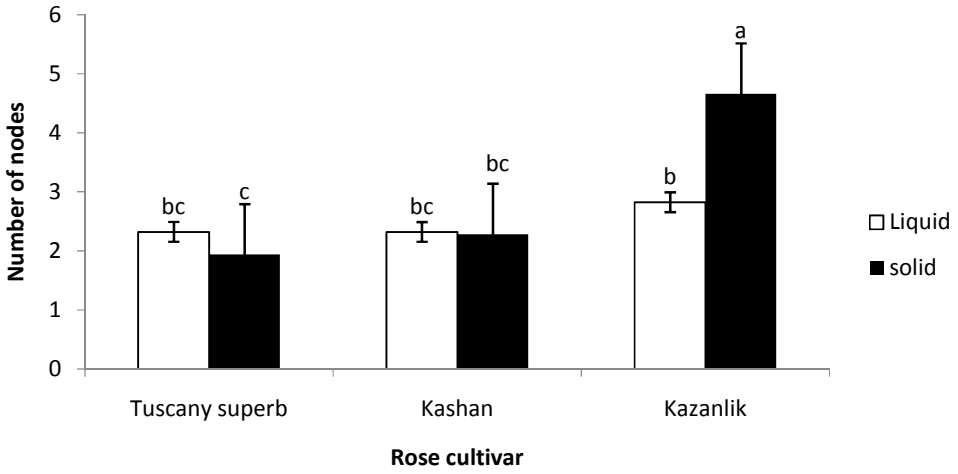

Fig. 8. Effect of medium type on *in vitro* number nodes of Damask rose cultivars.

Effect of plant growth regulators. Shoot length was not affected by used BAP concentration (Fig. 9). The number of leaves produced per propagule on MS supplemented with 0.25 mg l^{-1} BAP was significantly higher than the MS control while 0.5 mg l^{-1} BAP supplement used had no effect on number of leaves produced (Fig. 10).

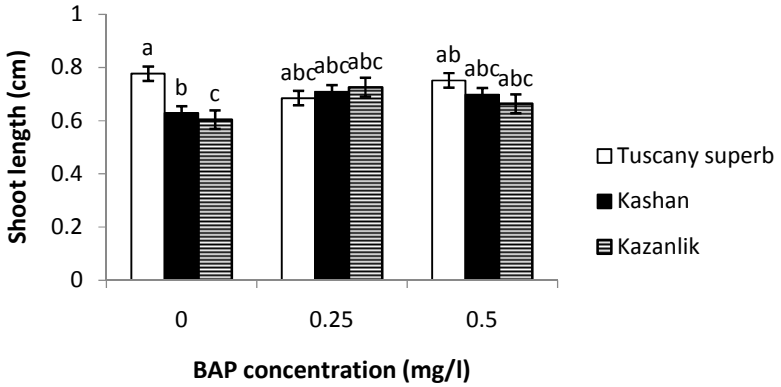

Fig. 9. Effect of BAP concentrations on shoot length of rose cultivars.

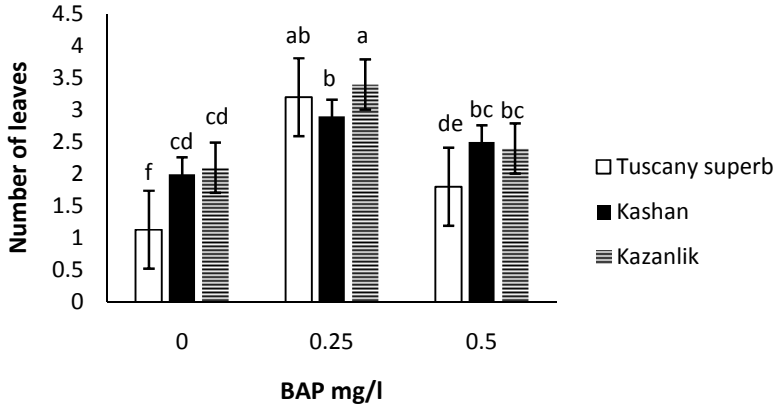

Fig. 10. Effect of BAP concentrations on *in vitro*leaf productionof Damask rose cultivars.

Higher BAP concentrations affected the rose cultivars differently such that Kazanlik produced more nodes with 2 mg l^{-1} and Tuscany superb with 1 mg l^{-1} BAP but it was the same in both concentrations for the Kashan cultivar. The highest number of nodes (4.2) was detected in Kazanlik with 2 mg l^{-1} BAP (Fig. 11). Using 1.5 mg l^{-1} BAP or thidiazoroun (TDZ) had no significant effect on number of shoot for any of the cultivars (Fig. 12). But regardless of cultivar, 1.5 mg l^{-1} BAP treatment significantly increased shoot length and number of nodes compared to the same concentration of TDZ (Table 5).

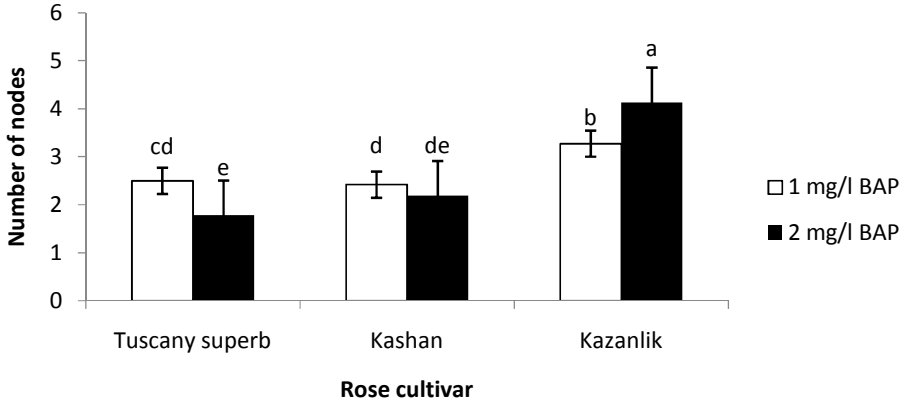

Fig. 11. Interaction of rose cultivar and BAP concentration on *in vitro* generated number of nodes.

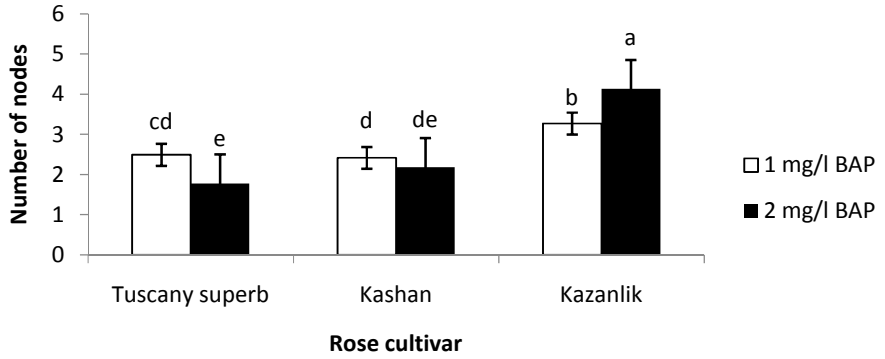

Fig. 12. Interaction of rose cultivar and cytokines type on number of *in vitro* generated shoots.

Table 5: Effect of cytokinin type on *in vitro* proliferation of rose.

Cytokinin	Shoot length (cm)	Number of nodes	Quality index
BAP (1.5 mg l^{-1})	1.84±0.11 a	1.82±0.31 a	1.57±0.12b
TDZ(1.5 mg l^{-1})	1.68±0.74 b	1.52±0.55 b	1.94±0.47a

Table 6: Effect of rose cultivar on its *in vitro* rooting.

Cultivar	Number of root	Rooting percent
Kashan	2.85 ±1.66b	%45±1.05 b
Kazanlik	2.13 ±1.11c	%30±2.14c
Tuscany	4.40±0.74 a	%55±1.7 a

Table 7: Effect of two medium culture on chlorophyll content.

Cultivar	MS	mMS
Kashan	0.10±001 d	0.15±004 c
Kazanlik	0.10±001 d	0.53±006 a
Gallica	0.14±002 c	0.20±003 b

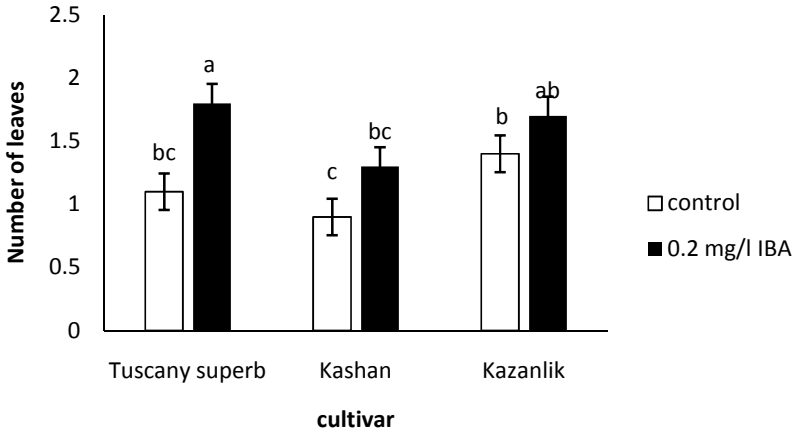

Fig. 13. Effect of IBA on *in vitro* number of leaf of Damask rose cultivars.

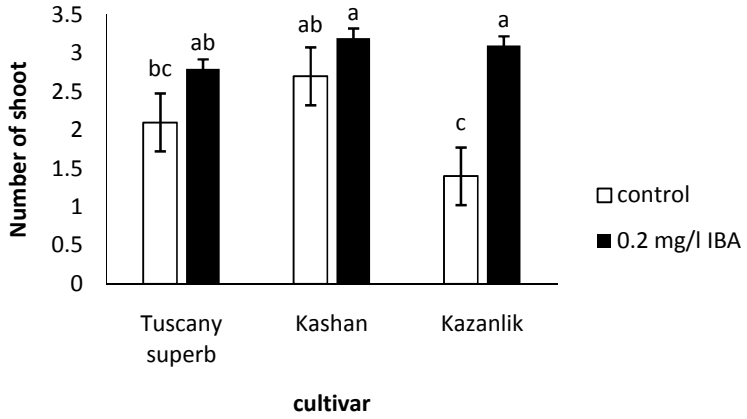

Fig. 14. Effect of IBA concentrations on *in vitro* shoot number of Damask rose cultivars.

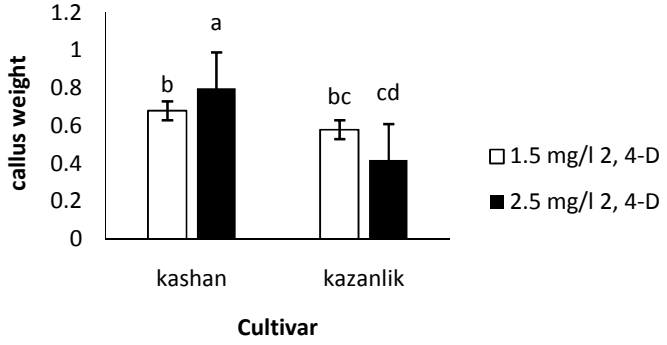

Fig. 15. Effect of 2,4-D on callus induction in two rose cultivar.

The combination of 2.5-3 mg l^{-1} BAP and 0.1 mg l^{-1} IBA was found to be the most suitable concentration for Damask rose propagation by Jabbarzadeh and Khosh-Khui (2005). Mahmoudi Noodezh *et al* (2012). Found that adding IAA either stimulated or inhibited shoot development in contrast to the effect of BAP. The best results on Damask rose propagation were achieved with MS medium containing 4 mg l^{-1} BAP plus 0.25 mg l^{-1} IAA (Bordbar 2005). Mahmoudi Noodezh *et al* (2012).

Indicated that using a hormone-free medium for the initial establishment stage (15 d), followed by transfer of explants, without separating the shoots, to the same medium supplemented with 4 mg l^{-1} BAP and 0.25 mg l^{-1} IAA resulted in the best response for propagation of the Damask rose. On the contrary, we found that low concentrations of BAP can significantly affect the number of shoots, shoot length and number of leaves. Since, high concentrations of hormones can adversely affect growth and cause mutations after many subcultures, it is better to use low concentrations.

C. Rooting and acclimatization

None of the rooting treatment significantly affected in vitro rooting of rose cultivars. But, rooting was significantly different depending on cultivar (Table 6). Such that, Tuscany Superb and Kashan root length and rooting percent were significantly higher than for Kazanlik (Fig. 16).

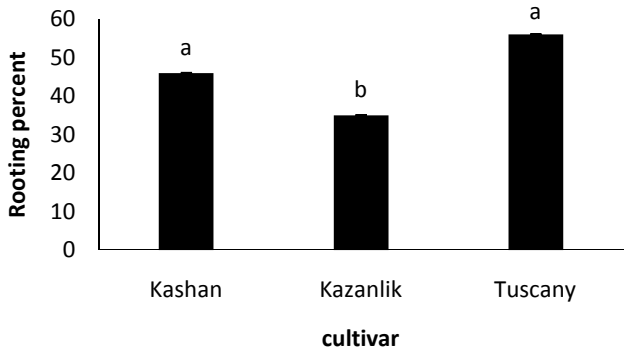

Fig. 16. Effect of Rose cultivar on rooting percent.

A close scrutiny of literature indicates that the rooting response with different auxins is cultivar dependent; also it is difficult to induce rooting in oil-bearing rose cultivars like Kazanlik (Kirichenko *et al.*, 1991). Further, Chu *et al.* (1993) reported that only shoots of *R. chinensis* cultured in liquid medium without BAP developed roots. However, in the present study, the experiments on root induction were done with great consistency and ease. Rooting of micro shoots is an important step for hardening and subsequent establishment in soil. The micropropagated roses are known to be a difficult system for hardening and acclimatization as they undergo rapid desiccation. Such plants are also susceptible to diseases due to high relative humidity (Messeguer and Melle, 1986). Moreover, it was reported by Tanimoto and Ono (1994) that multiple shoots of rose, established in liquid medium failed to acclimatize in soil. In the study of Kumar Pati *et al* (2005), these limitations were overcome by keeping the transplanted micro shoots in specially designed low-cost hardening chambers enriched with CO_2 and with high relative humidity, permitting up to 96.7% survival in *R. damascene* and 100% in *R. bourboniana*. We did not face this problem using jam jars.

Alderson *et al.* (1988) indicated that cellulosic sheets damped by liquid medium were successfully used as a platform for root induction. Kumar *et al.* (2001) studied the effects of agar and phytagel on rooting of Rosa damascene. When agar was used instead of phytagel as the solidifying agent, the number of roots per shoot increased. Mahmoudi Noodezh *et al.* (2012) observed the highest rooting rate in shoots cultured in liquid medium. Thus, they recommended using a liquid medium for root induction of in vitro-derived shoots of Damask rose. In contrast, we found that there is no difference in rooting of our studied cultivars of R. damascene and *R. gallica* in agar gelled and liquid media.

In conclusion, a practical economical, simple and efficient protocol has been developed for micropropagation of both *R. damascene* and *R. gallica* using liquid and agar gelled culture media. Importantly, a comparative analysis of in vitro performance of *R. damascena* and *R. gallica* indicates that efficiency of in vitro multiplication technique is strongly genotype dependent, allowing the selection of genotypes with high performance. Therefore, the overall results indicate that multiplication of these rose species can be performed efficiently by means of direct shoot proliferation using nodal segments from glasshouse grown plants on a commercial scale. Moreover, a modified MS medium containing 1450 mg l^{-1} NH_4NO_3 and 1700 mg $l^{-1}KNO_3$ resulted in the best growth in the proliferation phase.

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