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Research Article

Microspore Embryogenesis and Haploid Production in Tetraploid Rose Cultivars (*Rosa hybrida* L.) Using Anther and Shed Microspore Culture

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Abstract

The aim of the present research was to develop an efficient haploid production protocol in four tetraploid rose (*Rosa hybrida* L.) cultivars using anther and shed-microspore culture techniques. Buds containing a mixed population of mid to late uni-nucleate microspores were harvested from the main and lateral branches of donor plants and pretreated at 4°C for 7 days. The effects of plant growth regulators, induction medium (solid and double layer), stress pretreatment (heat and cold), and carbohydrate source (sucrose, glucose and maltose) were assessed on microspore embryogenesis induction in different genotypes. Induction of calli was enhanced significantly when MS medium containing BAP (0.5 and 1 mgl^{-1}) and IAA (4 and 8 mgl^{-1}) were used in 'Exotic' and 'Velvet' cultivars. The frequency of callus induction was higher in shed microspore culture compared to solid medium. Among various carbohydrate sources, sucrose-containing media showed a significant enhancement in callus formation. Heat (30°C for 3 days) and cold (4°C for 7 days) stresses could not improve the rate of callus induction in comparison with the control (25°C). microspore-derived embryos in 'Exotic' cultivar were formed in week 8 when cold-treated isolated microspores were cultured either in NN double layer medium supplemented with BAP (1 mgl^{-1}) + IAA (4 mgl^{-1}) or in MS double layer medium containing BAP (0.5 mgl^{-1}) + IAA (8 mgl^{-1}). Plantlets were regenerated when the microspore-derived embryos were transferred on to hormone-free MS or NN medium. Finally, 7 haploid plants were obtained via shed microspore culture.

Keywords: *Anther; Callus; Embryo; Rose (Rosa hybrida L.); Shed Microspore Culture*

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid
BAP: Benzylaminopurine
IAA: Indole-3-acetic acid
MS: Murashige and Skoogmedium
NAA: α-Naphthalene acetic acid
NN: Nitsch and Nitsch medium
Pg: Picogram

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PGR: Plant Growth Regulator
TDZ: Thidiazuron

Introduction

Haploid technology provides powerful tools for speeding up the breeding programs, improving selection efficiency, detecting linkage and gene interactions, QTL mapping, substitutions and chromosome addition lines [1]. Anther culture is one of the most commonly used methods for production of doubled haploid plants. However, DH technology is not well established in many economically important species such as *Rosa hybrida*. Roses are among the most popular ornamental crops all over the world. High degree of inter-cultivar sterility due to high degree of heterozygosity, different ploidy levels, perennial and woody nature and lack of inbred lines are some of the challenges in conventional breeding of roses [2]. [3] reported callus induction from anther culture of two cultivars of *R. hybrida* and observed a high frequency of diploid calli-derived from tetraploid species; however, no regeneration was occurred. To our knowledge, there is not any more report on rose anther/microspore culture, however in other genus of Rosaceae family such as sweet cherry and peach, haploid calli have been obtained using anther culture [4-6]. [7] Reported the production of two embryos from anther culture of *pyrus communis* cv. Le Lectier, but their origins were not determined and also regeneration was failed.

Anther culture has been successfully applied in many ornamental species e.g. *Lilium longiflorum* [8], *Pelargonium hortorum* [9], *Saintpaulia ionantha* [10] and *Tradescantia bracteata* [11] in order to produce doubled haploid plants, however its frequency was often low. Generally, various factors such as genotype, physiological state and growth conditions of donor plants, developmental stage of microspores, stress pretreatments, culture medium and *in vitro* conditions can affect the embryogenic response of anthers to *in vitro* culture [12].

The application of double layer medium in another culture stimulates anthers to dehisce and release their microspores into a liquid medium with high osmolarity. 'Shed-microspore culture' is a simple modification of another culture system. This method has been found to be successful in several species, including barley [13], wheat [14,15], tobacco [16], and pepper [17]. The presence of PGRs is necessary for production of microspore-derived embryos in the majority of plant species, particularly the recalcitrant ones [12]. The type and concentration of auxins seem to determine the pathway of microspore development [18]. According to Armstrong., *et al*. (1987) [19] and Liang., *et al*. (1987) [20], 2, 4-D induces callus formation and IAA or NAA promotes direct embryogenesis. However, the callus produced using only auxin is usually compact and non-embryogenic and has to be sub-cultured on a hormone-free or cytokinin containing medium for somatic embryogenesis [21]. The combination of zeatin and IAA in pepper (*Capsicum annum*) anther culture showed good results in both total embryo yield and the yield of normal-looking embryos (embryos with two cotyledons or with one visible cotyledon) [17].

For switching sporophytic pathway of developing microspores towards embryogenesis, many stresses such as high or low temperature, high humidity, water stress, anaerobic treatment, centrifugation, sucrose/nitrogen starvation, ethanol, X-irradiation, microtubule disruptive agents, electro stimulation, high medium pH, and heavy metal treatments were reported to be helpful [22].

In this paper, a shed-microspore culture method is reported for haploid plant production in four tetraploid *R. hybrida* cultivars for the first time. We compared solid and double layer media, different PGR combinations, various carbohydrate sources as well as temperature stresses (cold, heat) in order to induce embryogenesis and haploidisation in microspores of four cultivars of roses (*Rosa hybrida* L.).

Materials and Methods

Plant materials

Experiments were carried out at Agricultural Biotechnology Research Institute of Iran (ABRII) during the years 2013-2014. Rose cultivars 'Velvet', 'Elegant', 'Candy' and 'Exotic' were the test plants. Donor plants were grown in a greenhouse (in Hashtgerd, Karaj, Iran) at a day/night temperature of 18/21°C under fluorescent lamps (300 PPFD) with 16h light/8h dark photoperiod.

Anther culture

Flower buds were harvested when microspores were at mid to late uni-nucleate developmental stages. The majority of microspores were at the late uni-nucleate stage during early opening time of calyx, also few petals were visible on the buds. The developmental stage of the microspores was determined by staining with 0.1 mgl^{-1} 4', 6-diamidino-2- phenylindole (DAPI) [23] (Figure 1a). Buds were surface

Regeneration

Microspore-derived embryos were transferred to 10-cm Petri dishes containing MS or NN basal medium supplemented with 2% or 3% sucrose, 7-8 weeks following shed culture. All media were solidified with 0.6% Plant Agar. Cultures were kept under 16h light (60 $\mu mol\ m^2\ S^{-1}$) at 25 °C. After 3-4 weeks, shoots Exhibiting cotyledons and the first true leaf were transferred onto MS medium supplemented with 3% sucrose for plantlet regeneration.

Analysis of ploidy levels

For ploidy analysis, a piece of *in vitro* leaves (about 0.5 cm^2) was cut, then chopped together with a piece of parsley (*Petroselinum crispum*), 2C DNA= 4.45 pg, as an internal standard [24].The chopping was done by a razor blade in a Petri dish in 400 µl nuclei-isolation buffer [24]. Then the dye solution (1600 µl) containing 12 µl propidium iodide and 6 µl RNAse was added. The suspension was filtered through a 50-30 µm filters. The filtrate was incubated on ice for one hour, and the fluorescence intensity was measured by a flow cytometer (Partec PA2). The 2C DNA content of each sample was calculated by the formula (DNA amount (pg) = 4.474 p1/p2-0.012) reported by Yokoya., *et al*. (2000) [24].

Experimental design and assessment of the results

In each treatment, three 6-cm Petri dishes were used as 3 replications. In each Petri dish, 30 anthers were cultured randomly in solid or double layer media. The entire experiments were repeated at least for three times in two years. The percentage of callus induction (total calli per 100 anthers) was calculated 30 days after inoculation. Due to very low number of embryos, all figurers and tables were statically analyzed only for callus induction. Experiments were performed as factorial based on completely randomized design (CRD). Analysis of variance was performed using MSTATC software. Means were compared by the Least Significant Differences (LSD) method. Data transformation including arcsine square root was applied to normalize the distribution.

Experiments performed

Experiment 1: selection of cultivars and PGRs for callus induction in MS basal medium. In the first experiment, different PGRs including IAA (4 and 8 mgl^{-1}), BAP (0.5 and 1 mgl^{-1}), Kinetin (1.6 and 3.2 mgl^{-1}), 2, 4-D (2 and 5 mgl^{-1}), TDZ (0.1, 0.2 and 0.5 mgl^{-1}), and combination of IAA and BAP [4 mgl^{-1} IAA + 0.5 mgl^{-1} BAP, 8 mgl^{-1} IAA + 0.5 mgl^{-1} BAP, 4 mgl^{-1} IAA + 1 mgl^{-1} BAP, 8 mgl^{-1} IAA + 1 mgl^{-1} BAP] added in MS medium [25] and four rose cultivars were evaluated. All media contained 2% sucrose and solidified by 0.6% Plant Agar (Duchefa Biocheme).

Experiment 2: Assessment of different solid and double layer Culture media. For comparing solid and double layer media and testing the best PGRs combinations, different media (Table 1) with various combination of IAA + BAP [4 mgl^{-1} IAA + 0.5 mgl^{-1} BAP, 8 mgl^{-1} IAA + 0.5 mgl^{-1} BAP, 4 mgl^{-1} IAA + 1 mgl^{-1} BAP, 8 mgl^{-1} IAA + 1 mgl^{-1} BAP and a medium without PGRs as control] were tested in two cultivars of *R. hybrida* ('Velvet' and 'Exotic'). A double-layer medium system was used according to Supena., *et al*. (2006) [17]. The lower layer was MS solid medium consisted of 2% sucrose and 0.6% Plant Agar, while the upper layer was liquid Nitsch and Nitsch (NN) medium [26] contained 3% sucrose and solidified with 0.6% Plant Agar or AT3 [27] medium fortified with 3% sucrose and 10 gl^{-1} lactalbumin hydrolysate (AT3-SL) and solidified with 0.3% Phytagel. In double layer media, PGRs were added to the solid phase.

Solid layer	Liquid layer	Abbreviations
MS	-	MS
Nitsch & Nitsch	-	NN
1/2MS	-	1/2MS
AT3-SL	-	AT3-SL
MS	MS	MS/MS
MS	AT3-SL	MS/ AT3-SL
NN	NN	NN/NN
NN	AT3-SL	NN/AT3-SL
1/2MS	1/2MS	1/2MS/1/2MS
1/2MS	AT3-SL	1/2MS/AT3-SL
AT3-SL	AT3-SL	AT3-SL/AT3-SL

Means with different letters are significantly different at P=0.01 (F-test).

Table 1: *Different combinations of testing media (solid and double layer) in another culture of two rose cultivars ('Velvet' and 'Exotic').*

Experiment 3: *studying temperature stresses on two rose cultivars and media culture*. In this experiment, two stresses (4°C for 7 days/30°C for 3 days) applied to isolated anthers of two cultivars 'Velvet' and 'Exotic' were assessed in two different media (MS/AT3-SL and NN/ AT3-SL) with four combinations of IAA + BAP [4 mgl^{-1} IAA + 0.5 mgl^{-1} BAP, 8 mgl^{-1} IAA + 0.5 mgl^{-1} BAP, 4 mgl^{-1} IAA + 1 mgl^{-1} BAP, 8 mgl^{-1} IAA +1 mgl^{-1} BAP]. Room temperature (25°C) was used as a control. For cold pretreatment, flower buds were first put in a glass jar with screw lid on a plastic bag and stored at 4°C for 7 days before anther culture.

Experiment 4: Assessment of carbohydrate sources in NN basal medium. Different carbohydrate sources including glucose (20 gl^{-1}), sucrose (20 gl^{-1}), maltose (20 gl1), and their combinations were compared in NN media containing four combinations of IAA + BAP [4 mgl^{-1} IAA + 0.5 mgl^{-1} BAP, 8 mgl^{-1} IAA + 0.5 mgl^{-1} BAP, 4 mgl^{-1} IAA + 1 mgl^{-1} BAP, 8 mgl^{-1} IAA + 1 mgl^{-1} BAP] in two rose cultivars 'Velvet' and 'Exotic'.

Results

Effect of plant growth regulators on callus formation and embryogenesis in four cultivars. The first androgenic calli were observed about 4 weeks after anthers were transferred onto the culture medium. Results showed that different amounts of PGRs in (solid) MS medium did not induce any embryos in rose cultivars studied. The highest frequency of calli was observed in the MS medium containing IAA (0.5 and 1 mgl^{-1}) and IAA (4 and 8 mgl^{-1}) (Table 2). These combinations were applied in next experiments. There were significant differences among cultivars tested for callus formation. 'Velvet' and 'Exotic' cultivars induced higher percentage of calli (Figure 2). These two cultivars were subsequently included in further experiments.

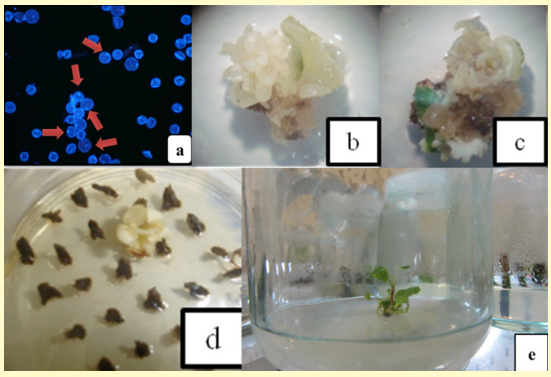
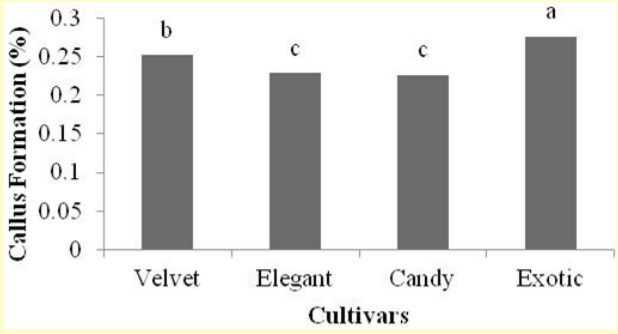


Figure 1: *The stages of plant regeneration of shed microspore culture. a- Microspore of Rosa hybrida cv. Exotic in late uni-nucleate stage (stained by DAPI) b: Embryonic calli obtained by anther culture in Rosa hybrida cv. Exotic c: callus regeneration d: Direct microspore embryogenesis in rose anthers after 8 weeks of culture e: Microspore-derived plantlet of rose obtained from embryo regeneration.*



Means with different letters are significantly different at P = 0.01 (F-test).

Figure 2: *Effects of four rose cultivars ('Velvet', 'Elegant', 'Candy' and 'Exotic') on callus formation.*

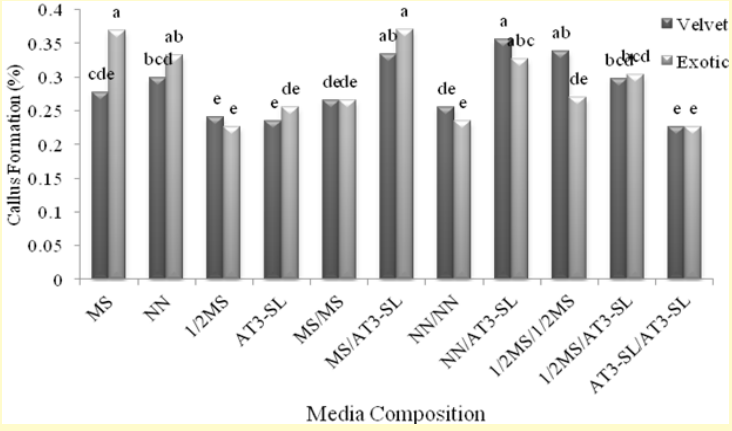
Effect of culture media on callus formation and embryogenesis. The effect of 11 different culture media on microspore embryogenesis showed that the highest callus formation was obtained in the MS (0.36%), MS/AT3-SL (0.37%), NN/AT3-SL (0.36%), and 1/2MS/1/2 MS (0.34%) media (Figure 3). However, NN and 1/2MS/1/2MS did not show any significant difference.

Generally, double layer media induced more calli compared to the solid media. The combination of BAP + IAA was important in callus formation, because, the absence of PGRs (as control) showed less formation (Figure 4). Embryo formation was observed only in the MS/AT3-SL medium supplemented with 0.5 mgl^{-1} BAP + 8 mgl^{-1} IAA (Figure 1d), but the frequency was very low (only 3 embryos), thus data analysis was not performed.

Plant Growth Regulators	Callus induction (%) Cultivars			
	Velvet	Elegant	Candy	Exotic
Control	0.25fg	0.23g	0.23g	0.23g
4 (mg/l) IAA	0.27efg	0.23g	0.23g	0.23g
8 (mg/l) IAA	0.25fg	0.23g	0.25fg	0.23g
0.5 (mg/l) BAP	0.23g	0.23g	0.23g	0.23g
1 (mg/l) BAP	0.23g	0.23g	0.23g	0.23g
1.6 (mg/l) K	0.27efg	0.23g	0.23g	0.23g
3.2 (mg/l) K	0.23g	0.25fg	0.23g	0.23g
14 (µmol/l)TDZ	0.23g	0.23g	0.23g	0.25fg
35 (µmol/l)TDZ	0.29def	0.23g	0.23g	0.23g
70 (µmol/l) TDZ	0.23g	0.23g	0.23g	0.25fg
2 (mg/l)2,4-D	0.23g	0.23g	0.23g	0.23g
5 (mg/l) 2,4-D	0.23g	0.23g	0.23g	0.23g
0.5 mg/l BAP+4 mg/l IAA	0.31de	0.23g	0.23g	0.50a
0.5 mg/l BAP+8 mg/l IAA	0.33cd	0.25fg	0.23g	0.35cd
1 mg/l BAP+4 mg/l IAA	0.25fg	0.23g	0.23g	0.37c
1 mg/l BAP+8 mg/l IAA	0.25fg	0.23g	0.23g	0.45b

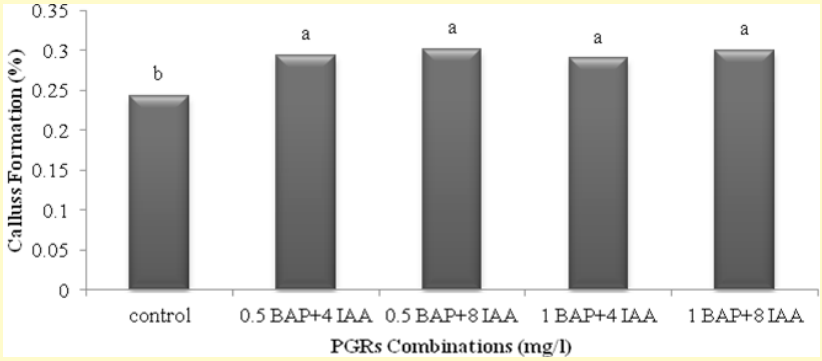
Means with different letters are significantly different at P = 0.01 (F-test).

Table 2: *Effects of plant growth regulators in MS medium on callus induction in four rose cultivars ('Velvet', 'Elegant', 'Candy' and 'Exotic').Means within a column followed by the same letter are not significantly different at P=0.01. Control: Culture without exogenous PGRs.*



Means with different letters are significantly different at P = 0.01 (F-test).

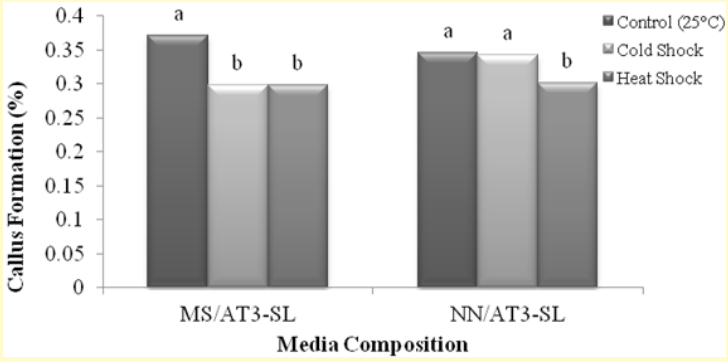
Figure 3: *Effects of different media on callus formation in two raised cultivars ('Velvet' and 'Exotic').*



Means with different letters are significantly different at P=0.01 (F-test).

Figure 4: *Effects of combined BAP and IAA on callus formation in Rosa hybrida cv. 'Exotic'.*

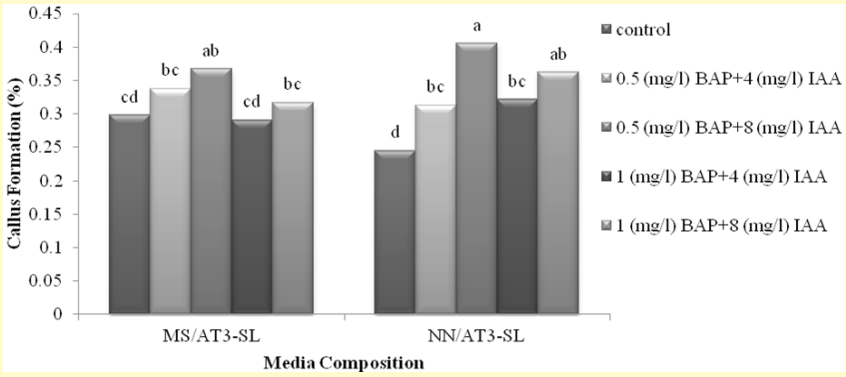
Effect of temperature stresses on embryogenesis and haploidisation of roses. Room temperature (25°C) could induce Calli in higher frequency compared to heat and cold stresses in (Figure 5). The lowest rate of callus was formed from anthers pretreated with 30°C for 3 days (Figure 5) in the both 'Velvet' and 'Exotic' cultivars (Figure 7), while control (25°C) in 'Exotic' cultivar induced more calli (Figure 7). BAP (0.5 mgl^{-1}) + IAA (4 mgl^{-1}) increased callus formation (Figure 6). Generally, induction of calli in all PGRs treatments was higher compared to control in NN/AT3-SL medium. But in MS/AT3-SL medium, the control and treatment with 1 mgl^{-1} BAP + 4 mgl^{-1} IAA induced the lowest frequency of calli.



Means with different letters are significantly different at P = 0.01 (F-test).

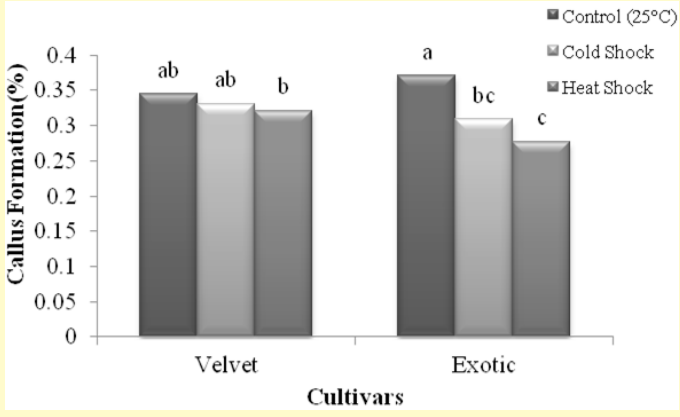
Figure 5: *Effects of two different media and temperature stresses (4º C for 7 days and 30º C for 3 days) on callus formation in two cultivars of rose ('Velvet' and 'Exotic').*

More importantly, for the first time 4 microspore-derived embryos were produced directly in the NN/AT3-SL medium containing 1 mgl^{-1} BAP + 4 mgl^{-1} IAA in cultivar 'Exotic', after seven weeks (one week at 4°C and the next 6 weeks at 25°C) (Figure 1d). The embryos were transferred to hormone-free NN medium.



Means with different letters are significantly different at P = 0.01 (F-test).

Figure 6: *Effects of different media with different combined BAP + IAA on callus formation in two cultivars of rose ('Velvet' and 'Exotic').*



Means with different letters are significantly different at P = 0.01 (F-test).

Figure 7: *Effects of cold and heat shocks on callus formation in two cultivars of rose ('Velvet' and 'Exotic').*

Effect of carbohydrate sources on embryogenesis and haploidisation. Among three carbohydrate sources (glucose, sucrose and maltose) supplemented in NN medium, the callus induction was significantly higher in sucrose-containing medium compared to media consisting of maltose or glucose (Table 3).

Ploidy analysis of regenerated plants

All of the obtained embryos (Figure 1d) formed shoots. Plant regeneration occurred after 3-4 weeks following embryo culture (Figure 1e). Ploidy levels of shoots regenerated from shed-microspore derived embryos were determined using flow cytometry. Results showed that all regenerated shoots were haploids (Figure 8).

Media Composition	Control	0.5 mg/l BAP+4 mg/l IAA	0.5 mg/l BAP+8 mg/l IAA	1mg/l BAP + 4 mg/l IAA	1 mg/l BAP + 8 mg/l IAA
NS	0.24gh	0.36bc	0.36bcd	0.35bcd	0.30defg
NS/NS	0.23h	0.26efgh	0.29defg	0.23h	0.23h
NS/AT3-SL	0.23h	0.31cde	0.35bcd	0.35bcd	0.45a
NM	0.23h	0.24gh	0.24gh	0.24gh	0.23h
NM/NM	0.23h	0.23h	0.23h	0.23h	0.23h
NM/AT3-SL	0.25fgh	0.25fgh	0.23h	0.23h	0.23h
NG	0.25fgh	0.24gh	0.24gh	0.24gh	0.23h
NG/NG	0.25fgh	0.27efgh	0.28efgh	0.27efgh	0.30defg
NG/AT3-SL	0.23h	0.23h	0.38b	0.26efgh	0.31cdef
NN(M+G)	0.26efgh	0.24gh	0.27gh	0.26efgh	0.24gh
NN(M+G)/NG	0.24gh	0.26efgh	0.25fgh	0.23h	0.25efgh
NN(M+S)	0.24gh	0.27efgh	0.25fgh	0.25fgh	0.25fgh
NN(M+S)/NN	0.25h	0.24gh	0.24gh	0.23h	0.25fgh
NN(G+S)	0.25fgh	0.25fgh	0.25fgh	0.25fgh	0.25fgh
NN(G+S)/NN	0.23h	0.26efgh	0.24gh	0.23h	0.23h

Means with different letters are significantly different at P = 0.01 (F-test).

Table 3: *Effects of various combinations of BAP + IAA on callus formation in two rose cultivars ('Velvet' and 'Exotic') (NS = NN with Sucrose (30 gl^{-1}), NM = NN with Maltose (30 gl^{-1}), NG = NN with Glucose (30 gl^{-1}), S = Sucrose, M = Maltose, G = Glucose) Means within a column followed by the same letter are not significantly different at P = 0.01. Control: Culture without exogenous PGRs.*

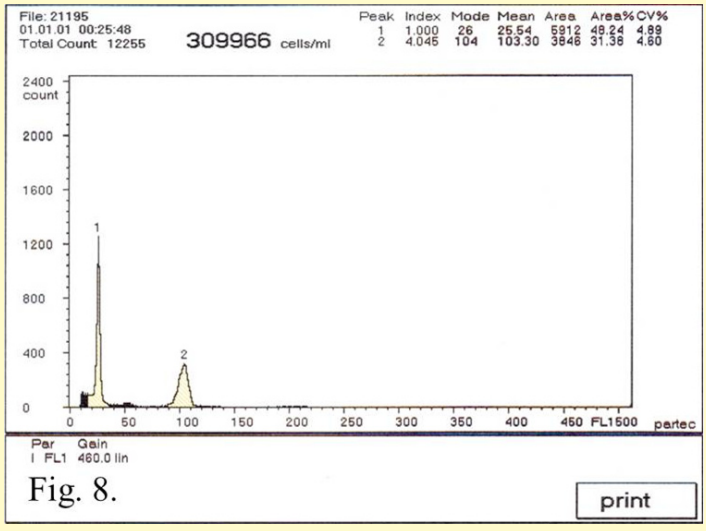


Figure 8: *Histogram of nuclear DNA contents obtained by flow cytometric analysis. Analysis of regenerated plants by "shed microspore culture" of 'Exotic'.*

Discussion

In this study, for the first time we developed a shed-microspore culture protocol in *Rosa hybrida* L. for haploid plant production. *Rosa hybrida* cvs. 'Exotic' and 'Velvet' were the most responsive cultivars, so they were selected for further experiments. Among the tested media, the double layer media induced more calli/embryos.

In most plant species, PGRs are necessary for microspore embryogenesis in anther culture [12]. The type and concentration of auxins determine the pathway of microspore development *in vitro*. In the present study, none of tested PGRs induced embryo (only callus formed). As presented in Table 2, among PGRs treatments, the combination of BAP and IAA was the best treatment for callus induction. It is reported that formation and growth of callus are mainly affected by the concentrations of auxins and cytokinins [28]. These results demonstrated that certain PGRs concentrations used in the embryogenesis induction medium were not suitable in calli/embryo induction. Dohm., *et al*. (2001) [29] and Hsia and Korban. (1996) [30] previously reported that callus was induced from leaf explants of *Rosa hybrida* cvs. 'Carefree Beauty' and 'Heckenzauber' with both auxin alone and in combination with cytokinin, whereas, in this research callus formation was induced only when auxin and cytokinin combined together in MS medium. Two cultivars, 'Velvet' and 'Exotic' exhibited the best response for callus formation among the four tested cultivars. Among the factors affecting embryogenesis, genotype is the most important. There are abundant papers reporting the genotype effect on the responses to anther culture. In our experiment, 'Elegant' and 'Candy' cvs. Showed the lowest percentage of callus therefore these cultivars were not used in the next experiments. Induction medium is another important factor in anther culture. MS and NN are two commonly used media in anther culture [12] and half-strength MS salt mixtures are convenient for Solanaceae [31]. Assani., *et al*. (2003) [32] reported that MS medium supplemented with 4.4 μM BAP + 2.3 μM IAA was appropriate for plant regeneration in anther culture of banana. MS basal medium supplemented with different combinations of PGRs, has been used in anther culture of several plant species such as *Tagetespatula* [33], *Rosa hybrida* (Tabaeezadeh and Khosh-Khui 1981) [3] and wheat [34]; therefore, it was applied in our experiment.

Our results showed that shed-microspore culture can be applied as a suitable system for haploid production in *R. hybrida* L. Generally, we observed embryos only in shed-microspore culture system. In double layer culture systems, the isolated anthers floated on the liquid upper layer medium and released their microspores into the medium. Predominantly, the embryos were induced from the released microspores, as reported in barley [13] and pepper [17].

Cold and heat shock produced more calli in comparison to non-treated cultures. A possible reason could be the application of non-proper duration of stress treatments. Stresses can switch gametophytic developmental pathway of microspores towards sporophytic. Heat and cold shocks are the most effective stresses inducing embryogenesis in microspores of anthers in many plant species [22]. 4°C and 10°C as cold and usually above 30°C as heat shock are used in different species [22]. It was suggested that cold pretreatment postpones degradation processes in the anther epidermis and endothelium membrane thus keeps microspores safe from toxic compounds released in the decaying anthers [35], so it was impressive in callus induction.

Results showed that sucrose in comparison to maltose and glucose was the best carbon source in the anther culture of two cultivars of rose ('Velvet' and 'Exotic'). Carbohydrate source is a key factor affecting microspore embryogenesis because of the osmotic and nutritional qualities [36]. The most common carbon source for anther culture is sucrose [37] and different concentrations of maltose were successfully replaced in some species such as wheat, triticale, rye and rice [38], but in our study it was not appropriate carbohydrate source in rose anther culture, in both solid and double-layer media. Raquin. (1983) [39] reported that fructose and glucose inhibited pollen embryogenesis in *Petunia* anther culture, as our results showed that glucose could not induce calli or embryos.

Cotyledonary-stage embryos were observed during seventh to eight weeks. Embryogenic calli derived from anther culture exhibited compact appearance and pale yellow-white or green coloration could preserve embryogenic potential for a long time. The morphogenic calli in rose appear friable and white same as Citrus calli reported by Germana (2006) [40]. The embryogenic calli differentiate into a clump of embryos. Supena., *et al*. (2006) [17] reported that in pepper only fully normal-looking embryos with two symmetrical cotyledon develop to viable seedlings. We observed the same results for calli which were not able to regenerate to plantlets.

Fortunately, we were able to reach embryos [but in low frequency (7)] which is valuable and outstanding results and could be an important initiation for future research. According to flow-cytometry results, all embryos obtained were haploids, indicating that the origin of embryos was from microspores. The embryos were mature enough to regenerate and produce plantlets [42-44] .

Conclusion

To conclude, we report haploid plant production in *Rosa hybrida* L. by shed microspore culture for the first time. The protocol can be used as a preliminary tool for producing haploid rose plants and it can be implemented for application in rose breeding programs, however, its efficiency needs to be improved in future studies. Our results showed that anther culture in rose follows a genotype-dependent manner. 'Exotic' and 'Velvet' cultivars showed the highest callus formation frequency on MS basal medium containing BAP + IAA. Only, one of the four tested cultivars ('Exotic') produced embryos. After transferring embryos to MS or NN media, embryos were developed into plantlets. Ploidy analysis proved that regenerates were haploids. Temperature stresses in comparison to control (25°C) produced less calli, which might be because of inappropriate type/period of stress duration. It was shown that sucrose was the best carbohydrate source in NN basal medium. Shed-microspore culture showed the best effect on callus/embryo formation. Generally, this research could be a basic study in rose anther/shed microspore culture for future studies.

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