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EFFECT OF PLANT GROWTH ADJUVANTS ON DIRECT REGENERATION OF MOHAMMADI FLOWER (*Rosa damascena* Mill.) USING THIN CELL LAYERING TECHNIQUE

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ABSTRACT

Regeneration is one of the most important methods in the massive proliferation of ornamental plants through micropropagation and genetic modification. Thin cell layering (TCL) technique, is a new introduction of micropropagation efficiency in producing optimized mass scale of crops. The horizontally placed TCL explants (tTCLs) was examined in Van der Salm (VS) basal medium in combination with different plant adjuvants (proline, glutamic acid, casein hydrolysate) to investigate their effect on callus induction and shoot regeneration of the explants. In order to produce healthy propagules of *Rosa damascena* Mill. on a large scale, a new cost-effective method of tissue culture has been established. The results indicated the positive effect of proline (0.5 gr/l) and glutamic acid (300 mg/l) in both callus induction and regeneration stage. Also, in rooting phase, different concentrations of IBA and NAA (0, 0.3, 0.6, 0.9 mg/l) in basal ½ Van der Salm (VS) medium were tested. It was observed that the best results were related to 0.3 mg/l IBA + 0.3 mg/l NAA in ½ Van der Salm (VS) medium.

Key words: thin cell layering (TCL), transversally, proline, glutamic acid, casein hydrolysate

INTRODUCTION

One of the strategic outlay of tissue culture technique, is to produce genetically uniform plants and facilities for pure lines, selection and cloning for different purposes such as genetic modification [Robert et al. 1987, 1992]. However, the cost of producing *in vitro* micropropagated plantlets is still high compared to those propagated naturally and traditionally. So, the new techniques with more efficient and cost-effective methods are needed for large scale production of commercial planting material especially for ornamental plants [Monja-Mio and Robert 2013].

The thin cell layer (TCL) technique uses thin cell tissue slice cuts [longitudinally (lTCL) or transversally (tTCL)] from the mother plants with the basis of in vitro or in vivo origin can be a suitable candidate to access this destination [Teixeira da Silva et al. 2007]. TCL was most likely never called thin tissue layer (TTL), possibly because as mentioned before two types of TCLs exist: tTCLS and lTCLs, which tTCLs represent a set of multiple tissues, while the lTCLs represent a single set of tissues [Teixeira da Silva 2008]. There is an inherent contradiction between the two terms TCL and TTL, in any TCL technique, the emphasis is on "thin" although the originator of the technique (Tran Thanh Van) which defined this technique with tobacco plant for the first time with the initial intended

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focus on the creation of the unique, homogeneous, and uniform layer of cells that could control plant tissue growth. Thin cell layering technique is one of the most effective remedial methods for regenerative purposes, due to its ability to regenerate plants from processional calluses is one of the fundamental stages in micropropagation protocol set up for genetic engineering. Therefore, the achievement of the plant regeneration protocol using a thin cell layer of cells has significant importance in research projects [Azadi et al. 2017]. In this technique, plant cells improve regeneration ability by establishing a balanced relationship between plant hormones and growth promoters in the culture medium, so it is considered as an effective step in genetic modification of plants [Nhut et al. 2006].

The efficiency of callus formation and plant regeneration depends on several factors such as plant genotypes, tissue culture technique, developmental stage, type of explant and more important combination of culture medium [Vasil and Vasil 1986]. It seems that the weak potential of plant cells in regeneration and lack of proper regeneration protocol for many modern cultivars is an important limitation in plant breeding programs [Haque et al. 2015]. Recently some reports have been mentioned on the effect of plant adjuvants like $AgNO_3$, L-proline, L-glutamine, casein hydrolysate, biotin, thiamine, etc. on different stages of plant micropropagation [Bora et al. 2019].

TCLs have been successfully used for the regeneration of several ornamentals such as orchids, lilies, African violets, begonias, etc. [Teixeira da Silva and Dobranszki 2013]. *Rosa damascena* Mill. is one the medicinal ornamental plants with aromatic important value in the world's Aromatic industry especially in Iran's. As mentioned before, there had been several research projects on micropropagation of different cultivars of *Rosa damascena* Mill. [Kumar et al. 2001, Jabbarzadeh and Khosh-Khui 2005, Nikbakht et al. 2005, Pati et al. 2005, Mahmoudi Noodezh et al. 2012, Alsemaan et al. 2013], but there isn't any attempt on applying TCL technology on regeneration of this important native plant of Iran as a new recourse in order to increase the regeneration rate. Therefore, this is the first recital of an efficient protocol to introduce the effect of different plant growth promotors on increasing the rate of microprogramed plantlets of this valuable native plant from thin stem segments that enhances the production of elite clonal. *Rosa damascena* Mill. plants previously generated through micropropagation of adventitious shoots.

MATERIALS AND METHODS

Plant materials and culture media for Initiation stage. The healthy bases of *R. damascena* with high levels of essential oil and strong growth were selected from Farokhod village from Khorasan Razavi functions for collecting herbs. One-year old shoots were considered as suitable specimen. After transferring the branches to the laboratory, lateral sprouts were cut and disinfected. For the sterilization stage, 1 cm axillary bud explants were washed one hour with running tap water, surface sterilized with Benomyl fungicide (1%) for 15 min, 70% ethanol (30 s), sodium hypochlorite (2.5%) for 15 min, 0.1% mercury II chloride ($HgCl_2$) (10 min) and then were washed 3 times with double-distilled water. The sterilized explants were cultured in Van der Salm (VS) medium containing 300 mg/l silver nitrate ($AgNO_3$) and 300 mg/l ceftotaxim, which were used to control inner bacterial infections. The reason for using high concentration of silver nitrate and antibiotic in the culture medium is the accumulation of internal bacterial agents in the autumn and winter seasons in the natural habitat of this plant [Davoudi Pahnekolayi et al. 2015].

Applying TCL technique from in vitro explants at regeneration phase. After culture initiation and growth healthy axillary branches, TCL technique was applied. Plantlets, 1–2 cm (10–20 mm) tall with the stem diameter of 0.1–0.3 cm (1–3 mm), were used as a source of TCL explants. In this research VS medium which contains FeEDDHA chelate of iron instead of MS medium with FeEDTA chelate was applied. The outer leaves were removed from the stems and they were cut transversally into thin segments in 0.5–1.0 mm thickness (tTCLs). About ten slices were obtained from one shoot. The TCLs were cultured in induction medium consisting of basal VS salts supplemented with 2 mg/l 6-benzylaminopurine (BAP) + 0.1 mg/l 1-naphthaleneaceticacid (NAA) + 25 mg/l $AgNO_3$ and different concentration of proline (0, 0.5, 1 gr/l), glutamic acid and casein hydrolysate (0, 300, 600 mg/l). All culture media were adjusted to a pH of 5.75 with a 0.1 N solution of either HCl of NaOH and

sterilized at 121°C for 15 min. For the induction phase, the cultures were incubated in light/dark photoperiod (16/8 h) at 25 ±2°C for 60 days [Monja-Mio and Robert 2013]. The experimental design was a completely randomized design (CRD) with 7 treatments and 3 replications (10 explants replication). Different characters related to induced callus and regenerated plantlets were measured in this stage.

Rooting and acclimatization phase. In the rooting stage, various concentrations of plant growth regulators (PRGs) were used in two different culture media to study the rooting characteristics of regenerated shoots. The appropriate regenerated plantlets isolated and cultured in ½ VS medium containing different concentrations of NAA and indole-3-butyric acid (IBA) (0, 0.3, 0.6, 0.9 mg/l) supplemented with 25 mg/l $AgNO_3$. Also, the combination of 0.3 mg/l NAA + 0.3 mg/l IBA was investigated. The factorial experiment was done in completely randomized design (CRD) with 8 treatments and 3 replications. After 30 days, rooting characters (root number, root length and rooting percentage) were recorded and well-rooted plantlets were washed to remove residual nutrients and agar and then transferred to plastic bags with a mixture of cocopeat: perlite (1 : 1) for acclimatization stage. The bags were transferred to the greenhouse after one week and survival rates were evaluated after 30 days [Teixeira da Silva and Malabadi 2012].

Statistical analysis

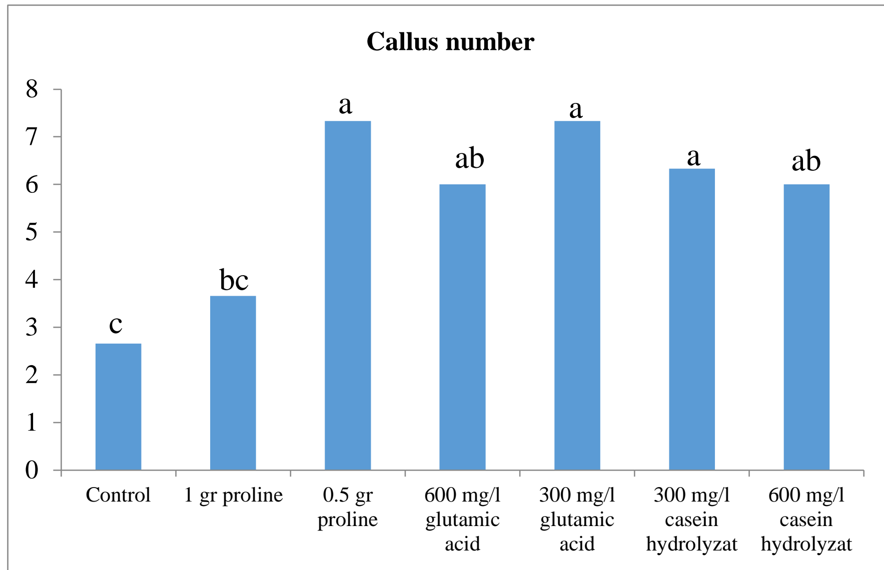
All the data were analyzed with SPSS (19) statistics program. The analysis of variance (ANOVA) was used to calculate statistical significances using LSD test at $p \leq 0.05$ level.

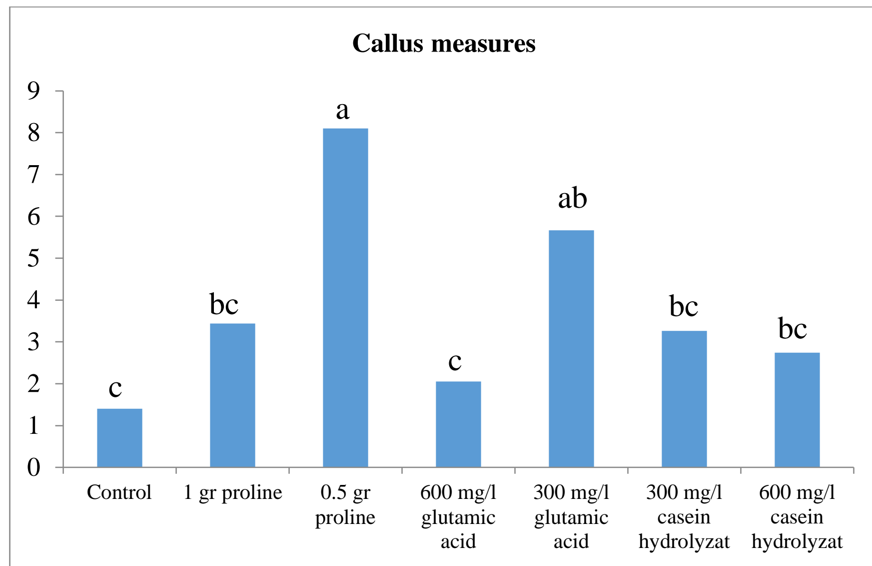
RESULTS AND DISCUSSION

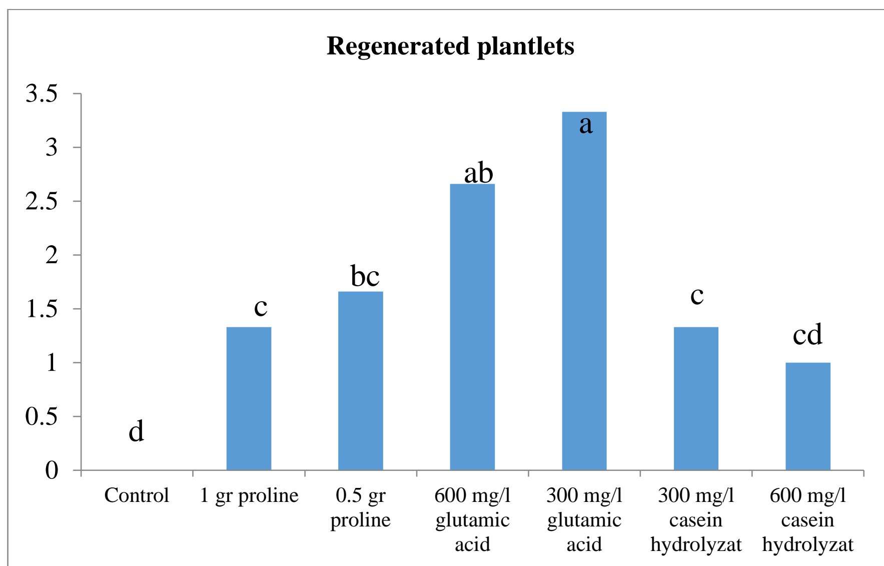
Regeneration stage from TCLs. There was a significant difference between treatments in regeneration step from non-response to callus formation and stem regeneration ($p \leq 0.05$) (Tab. 1). The fact that stem tissues have the ability to induce callus and regenerate shoots has been proved before [Monja-Mio and Robert 2013], but the type and composition of the culture medium is so determinant. The results of this study showed that in control treatment without the presence of growth adjuvants, shoot regeneration was not performed. Although, the callus induction was observed. Primary calluses with whiteish-yellow color in all response treatments were formed one month after cul-

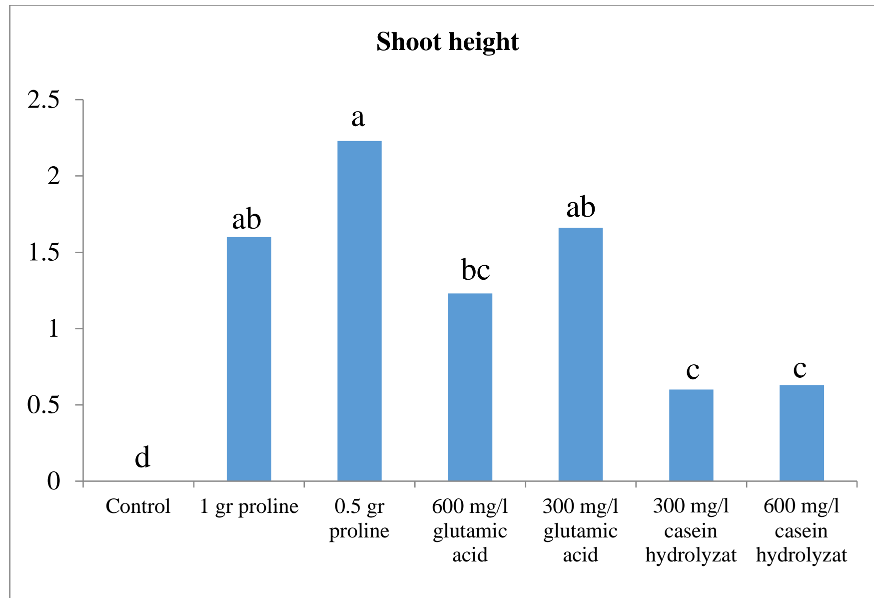
Table 1. Analysis of variance (ANOVA) of *Rosa damascena* Mill. TCLs in regeneration stage ($p \leq 0.05$)

Parameters	SPSS ANOWA analysis	Sum of squares	df	Mean square	F	Sig.
Callus number	between groups	57.619	6	9.603	5.307	.005
	within groups	25.333	14	1.810		
	total	82.952	20			
Callus measures	between groups	97.074	6	16.179	5.886	.003
	within groups	38.482	14	2.749		
	total	135.556	20			
Regenrated plantlets	between groups	21.619	6	3.603	9.458	.000
	within groups	5.333	14	.381		
	total	26.952	20			
Shoot length	between groups	7.851	6	1.309	5.315	.005
	within groups	3.447	14	.246		
	total	11.298	20			
Leaf number	between groups	205.333	6	34.222	3.223	.033
	within groups	148.667	14	10.619		
	total	354.000	20			


Callus number
8
7
6
5
4
3
2
1
0
c
bc
a
ab
a
a
ab
Control
1 gr proline
0.5 gr proline
600 mg/l glutamic acid
300 mg/l glutamic acid
300 mg/l casein hydrolyzat
600 mg/l casein hydrolyzat


Callus measures
9
8
7
6
5
4
3
2
1
0
c
bc
a
c
ab
bc
bc
Control
1 gr proline
0.5 gr proline
600 mg/l glutamic acid
300 mg/l glutamic acid
300 mg/l casein hydrolyzat
600 mg/l casein hydrolyzat


Regenerated plantlets
3.5
3
2.5
2
1.5
1
0.5
0
d
c
bc
ab
a
c
cd
Control
1 gr proline
0.5 gr proline
600 mg/l glutamic acid
300 mg/l glutamic acid
300 mg/l casein hydrolyzat
600 mg/l casein hydrolyzat



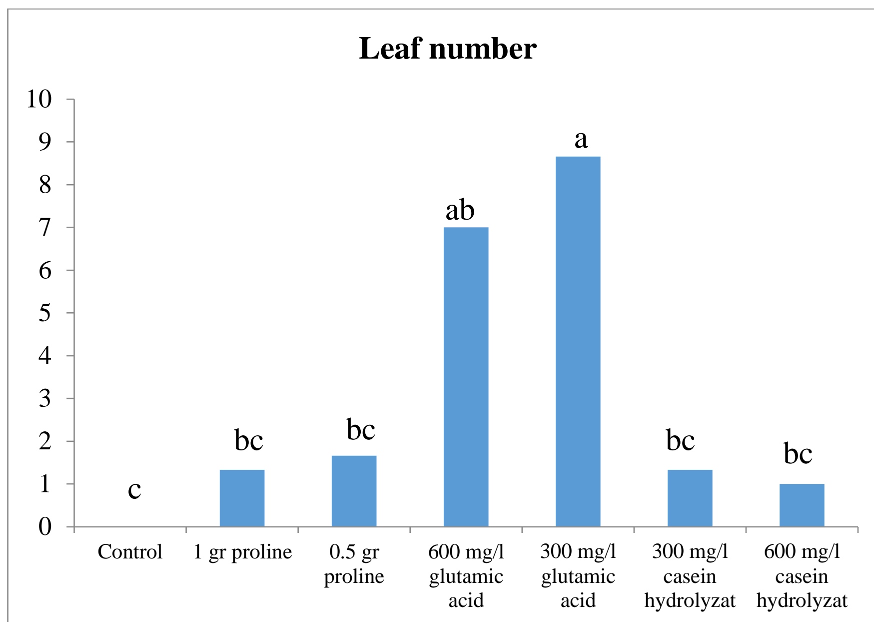


Fig. 1. The effect of different amino acids on callus induction and regeneration of *Rosa damascena* Mill. From tTCLs *in vitro* condition: (A) callus number, (B) callus measures, (C) regenerated plantlet, (D) shoot height, (E) leaf number

tivation, especially in low-concentrations of growth stimuli, which is one of the benefits of TCL culture rather than other techniques, surprisingly by increasing concentrations, improving in callus induction was not achieved. These calluses turned to green just before the regeneration process. The highest amount of callus induction (7.33) was observed in proline (0.5 gr/l), glutamic acid (300 mg/l) and casein hydrolysate (300 mg/l) respectively and the lowest in control treatment (2.66) (Fig. 1A). However, the results proved the appropriate application of the tTCL technique for callus induction in *R. damascena* like in saffron [Azadi et al. 2017]. The application of glutamic acid in several plants has shown different results. In *R. damascena*, callus formation, shoot regeneration and increased leaf number were significantly affected by glutamic acid, while in *Chrysanthemum hybridum* 'Yoder white', *Gerbera jamesonii* 'Harriet' and *Cordyline frutycosa* 'Kiwi', positive reactions has not been significantly reported [Samarina et al. 2016]. Although proline, glutamic acid and casein hydrolysate have a significant effect on indirect regeneration of *R. damascena* by using TCL technique, proline (0.5 gr/l) has been shown better results in increasing the callus volume and the height of regenerated plantlets (Fig. 1B, 1D), while glutamic acid (300 mg/l) was effective in increasing the number of regenerated shoots and their leaf number (Fig. 1C and E) in comparison to others. Therefore, it can be argued that even low proline concentrations (0.5 gr/l) in combination with glutamic acid (300 mg/l) can have a positive effect on the regeneration of this rose species and can be suggested as an efficient protocol on a large-scale production of this ornamental-medicinal native plant.

Application of different amino acids as one of the nitrogen sources in the culture medium can have a complementary impression on different stages of plant growth [Thorpe et al. 2008]. In organogenesis of pineapple, 1.2 gr/l glutamine in the culture medium Increased the level of micropropagation competence up to 70% [Hamasaki et al. 2005], and 1.5 gr/l glutamate in palm, resulting in enhanced somatic embryogenesis capacity [Mariani et al. 2014]. On the other hand, regarding the effective application of different amino acids in combination with growth regulators for in vitro propagation of several horticulture crops [Siwach et al. 2012, Medza Mve et al. 2013], but so far in regeneration of ornamental plants such as *R. damascena*, using these materials as growth adjuvants for optimization are not mentioned before. Therefore, it is the first report of the inquiry of amino acids on regeneration of *Rosa damascena* plantlets from TCLs, it revealed two points: first, the positive action of growth promotors in combination with PGRs and introducing tTCLs as suitable and cost-effective explants for mass micropropagation of *Rosa damascena*, which was achieved successfully.

Casein hydrolysate (CH) is one of the most widely used growth stimuli in plant tissue culture for various purposes especially somatic embryogenesis [Pandey and Tamta 2014]. The purpose of this nitrogen combination in tissue culture differs according to the plant type and its developmental stage [Pandey and Tamta 2014]. So that there are various effects, such as escalating the rate of green leaves and preventing the production of yellow leaves which is one of the main problems in rose tissue culture caused by the undesired ethylene production, the elimination of callus formation in inadvertent conditions, and the improvement of plant micropropagation [Davoudi Pahnekolayi et al. 2017]. In the present study, the beneficial effects of this substance have been evidenced in callus and regeneration, but in comparison with proline and glutamic acid it is in third place. CH is a rich wellspring of phosphorus, calcium, vitamins and several micronutrient compounds. It is reported that it is a mixture of about 18 different amino acids, which have a noticeable effect on plant growth. Perhaps this is why, in many cases, it is proposed as a substitute for gibberellic acid (GA_3) to reduce the problem of leaf yellowing in vitro condition [Bhatia and Ashwath 2008].

The use of L-proline in callus establishment and regeneration experiments showed that it plays a complementary role with plant growth regulators (PGRs) particularly in embryonic ones, because in many cases the presence of proline even in low concentrations can have a conspicuous impressiveness than the usage of PGRs alone [Chowdhry et al. 1993]. As it is described in this study, 0.5 gr/l proline in combination with 2 mg/l BAP + 0.1 mg/l NAA + 25 mg/l $AgNO_3$ refined the callus induction and shoot regeneration. So far, there is a lot of research about optimizing different growth regulators at various levles of growth and development of plant cells, but one of the goals

Table 2. Effect of PGRs type on some traits of *Rosa damascena* in rooting stage

PGRs Type	Root number	Root length (cm)	Rooting percentage (%)
Control	0 b	0 b	0 b
NAA	2.4 ±0.44 ab	0.77 ±0.06 ab	27.15 ±4.93 ab
IBA	3.5 ±0.88 a	1.68 ±0.29 a	38.88 ±9.81 a

Different letters indicate significant difference according to Duncan test ($p < 0.05$)

Table 3. Effect of PGRs concentrations on rooting traits of *Rosa damascena*

Concentrations (mg/l)	Root number	Root length (cm)	Rooting percentage (%)
0	0 c	0 b	0 c
0.3	2.66 ±0.66 ab	0.84 ±0.03 ab	29.62 ±7.4 b
0.6	1.83 ±0.16 b	1.041 ±0.05 ab	20.36 ±1.85 ab
0.9	4.83 ±1.57 a	1.9 ±0.61 a	53.69 ±17.54 a

Different letters show significant differenced according Duncan test ($p < 0.05$)

Table 4. PGRS type and concentrations effect on ANOVA (mean of square) analysis of some traits of *Rosa damascena* in rooting stage ($p \leq 0.05$)

Parameters	SPSS ANOWA analysis	Sum of squares	df	Mean square	F	Sig.
Root number	between groups	138.000	7	19.714	33.796	.000
	within groups	9.333	16	.583		
	total	147.333	23			
Root length	between groups	19.602	7	2.800	62.084	.000
	within groups	.722	16	.045		
	total	20.323	23			
Rooting percentage	between groups	17034.889	7	2433.556	33.794	.000
	within groups	1152.181	16	72.011		
	total	18187.070	23			

Table 5. Effect of treatments (PGRs and concentrations) on rooting traits of *Rosa damascena*

Treatments	Root length	Root number	Rooting percentage
Control	0 e	0 d	0 d
0.3 mg/l NAA	0.83 ±0.06 cd	4 ±0.57 b	44.44 ±6.41 b
0.6 mg/l NAA	0.94 ±0.02 c	2 ±0 c	22.22 ±0 c
0.9 mg/l NAA	0.53 ±0.03 d	1.33 ±0.33 cd	14.81 ±3.7 cd
0.3 mg/l IBA	1.5 ±0.28 b	2.66 ±0.88 c	29.62 ±9.7 c
0.6 mg/l IBA	0.85 ±0.05 cd	1.33 ±0.33 cd	14.81 ±3.7 cd
0.9 mg/l IBA	1.13 ±0.08 bc	1.66 ±0.33 c	18.51 ±3.7 c
0.3 mg/l NAA + 0.3 mg/l IBA	3.26 ±0.14 a	8.33 ±0.33 a	92.58 ±3.7 a

Different letters show significant differenced according Duncan test ($p < 0.05$)

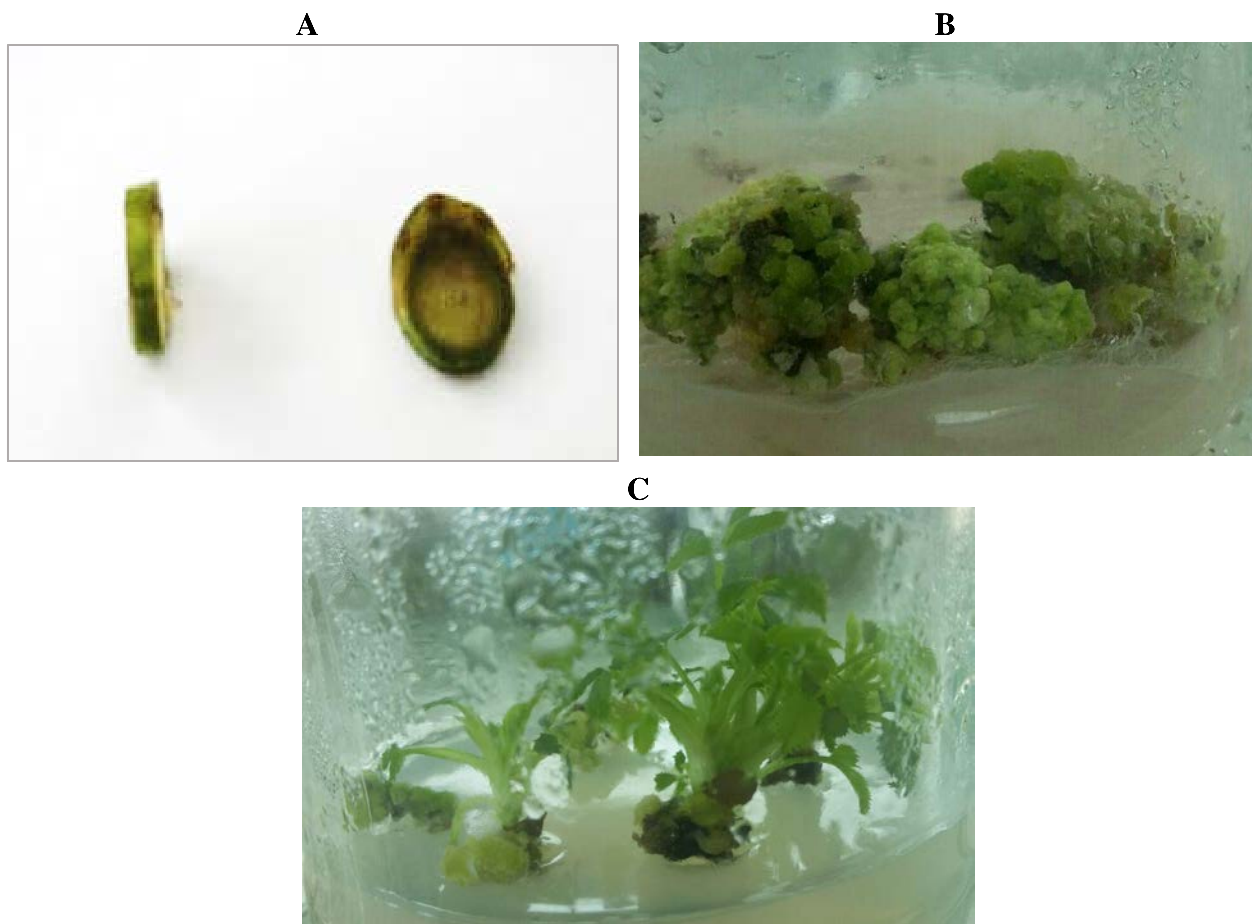


Fig. 2. Effect of plant growth adjuvants on tTCLs of *R.damascena* Mill. *in vitro* condition. (A) tTCL explants, (B) callus induction, (C) regenerated plantlets

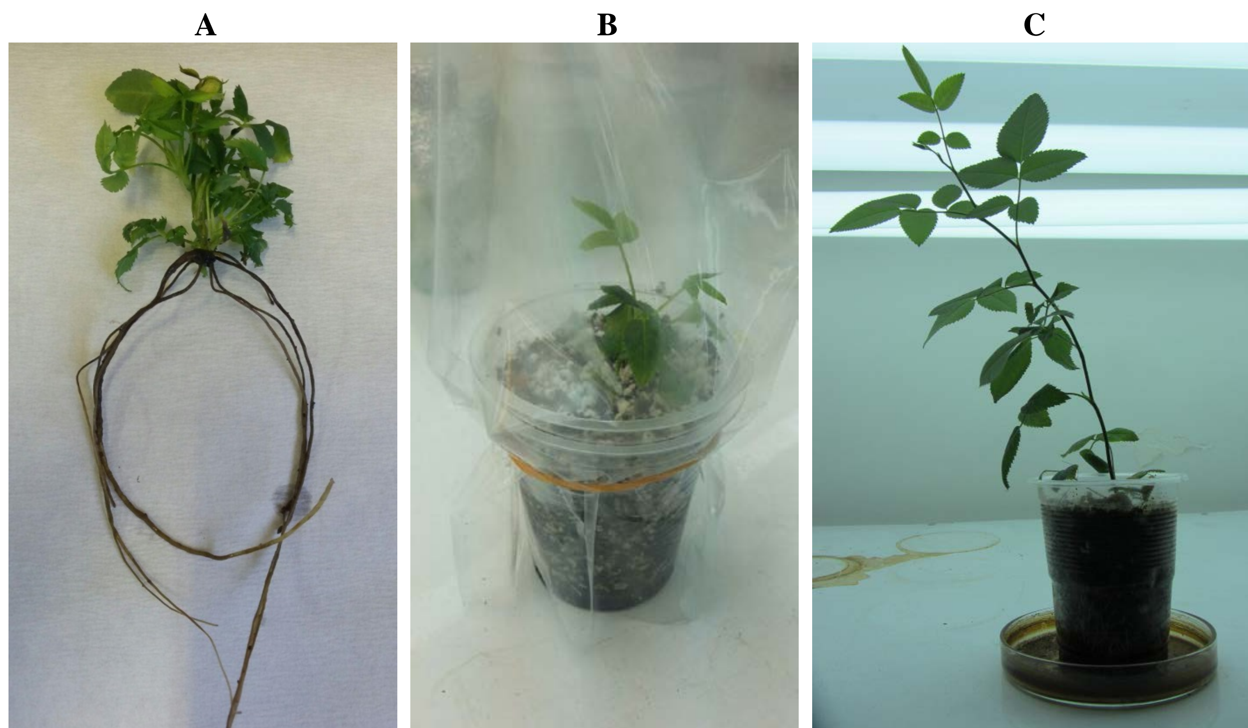


Fig. 3. Effect of plant growth adjuvants on of *Rosa damascena* Mill. plantlets. (A) rooted plantlet, (B) acclimatization stage, (C) acclimatized plantlet

of micropropagation, especially for certain plants, is the maximum and mass production in the shortest possible time and unfortunately *R. damascena* explants demonstrated lower levels of proliferation than other species of roses, before [Armstrong and Green 1985, Vasil and Vasil 1986, Trigiano and Conger 1987, Ozawa and Komamine 1989]. Therefore, increasing its multiplication rate due to its strategic importance in Iran is worth to be considered. Due to the high supply of nitrogen compounds and several amino acids in Proline and glutamic acid with the absence of prolonged poisoning, they can stimulate the growth of plant cells and increase the amount of efficiency.

The achievement of immediate and direct shoot regeneration for TCLs of nodal segments declared another antidote for mass propagation of commercial crops especially important ornamental plants like *R. damascena*. This novel approach in tissue culture technique is more suitable than other in vitro culture methods. These results are in accordance with other previous studies carried out on different species [Vyas et al. 2010. Nhut et al. 2001]. In begonia species very high number of regenerated plantlets was reported by using tTCLs [Nhut et al. 2013]. Here, it is relevant to state that the induction from nodal segments has a shorter and more rapid response period as compared to the tTCL experiments however the total number of regenerated shoots obtained is higher and more cost-effective in TCL technique (Fig. 2).

Rooting and acclimatization stage. PGRs type on rooting characters revealed significant differences between ($p \leq 0.05$) (Tab. 2). The results exhibited that the presence of the growth regulators has significant effect on rooting stage. Also, the analysis declared significant influence of NAA and IBA in comparison control, while IBA showed better result in all investigated characters (Tab. 2). According to different concentrations of PGRs, the best results were related to 0.9 mg/l in all characters (Tab. 3). As the effect of both PGRs and concentrations have rightful role for rooting stage, the ANOVA analysis showed significant difference in all the mentioned traits (Tab. 4). According to the results the maximum root number (8.33), root length (3.26 cm) and rooting percentage (92.88%) were related to 0.3 mg/l NAA + 0.3 mg/l IBA (Tab. 5).

In micropropagation technique, different auxin combinations are used for several purposes like rooting one, which is one of the important phases of *in vitro* propagation. The application of synthetic auxin i.e. IBA might have increased the biosynthesis of IAA or could have synergistic effect on the action of IAA [Dixon and Gonzales 1993]. Another possible reason for efficient and rapid rooting of *R. damascena* plantlets, might be the involvement of IBA in ethylene biosynthesis [Arteca and Arteca 2008]. It has been reported auxin induced ethylene may increase adventitious root formation instead of action of auxin itself [Mudge 1989], also it should be mentioned that presence of $AgNO_3$ in the culture medium in all *in vitro* stages will control the negative effect of ethylene on yellowish of leaves and in many cases will help the initiation of roots in rooting phase [Davoudi Pahnekolayi et al. 2017].

Rather than the effect of different PGRs, other substances which are added to the culture medium will have a positive effect on rooting. In MS medium, FeEDTA is the major source of iron for plant growth, which induces many problems such as precipitation, decrease of Fe availability and production of toxic compounds [Pati et al. 2005]. Also, in some cases, it inhibits rooting occurred [Rout and Jain 2004]. The interaction of PGRs like IBA with iron chelate in VS medium may have a stimulator effect on rooting, which is approved before [Sadeghi et al. 2015]. Also, the application of ethylene biosynthesis inhibitors or ethylene absorbents like silver nitrate will decrease the harmful effect of ethylene and increase the root length quality which is an important point for acclimatization stage [Sharafi 2010].

Acclimatization of the rooted plantlets was rapidly managed at ±24°C and 80% humidity for the first week and then the acclimatized plantlets transferred to the greenhouse with controlled humidity (40%) and temperature (±26°C). 95% of the micropropagated plants were survived successfully (Fig. 3).

CONCLUSIONS

To conclude, the success and rapid shoot regeneration of *Rosa damascena* Mill. From tTCL explants introduces an efficient way to propagate this valuable native plant. Such small explants are relatively uniform, very sensitive, cost-effective and have rapid responses to different concentrations of adju-

vants especially amino acids. Following the results, the positive effect of proline (0.5 gr/l) and glutamic acid (300 mg/l) in both callus induction and regeneration stage has been approved. Moreover, it was observed that the combination of 0.3 mg/l IBA+0.3 mg/l NAA in ½ Van der Salm (VS) medium can be suggested as the best treatment for rooting of *Rosa damascena* Mill. plantlets.

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