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Research Article

Evaluation of the effect of cytokinins, thiamine (VB1), brassinosteroids, and auxins on organogenesis potential, and protoplast isolation in *Rosa damascena* Mill *in vitro* condition

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Abstract

Damask rose (*Rosa damascena* Mill.) is one of the ornamental and medicinal plants. In this study, shoot explants were cultured on MS medium with different concentrations of IAA, 2, 4-D, BAP, Kin, Br (brassinosteroids), and Vitamin B1 (VB1) in two group tests. Protoplasts were isolated from leaves with the enzymes and osmosis of sucrose. The results were studied, after the fourth week. In test 1, the results showed that 1 mg l^{-1} IAA with 0.1 mg l^{-1} Br had the highest rooting (63.33%), 1 mg l^{-1} IAA with 0.1 mg l^{-1} VB1 had the maximum shoot formation (66.67%), and 1 mg l^{-1} IAA with 0.1 mg l^{-1} Kin had the highest callus production (19%). The concentration of 0.1 mg l^{-1} IAA with 1 mg l^{-1} of VB1 was suitable for rooting (40%), shoot formation (50%), and low callus (10%) simultaneously. The highest rooting (73.33%) was obtained by using 4 mg l^{-1} 2, 4-D with 0.4 mg l^{-1} BAP. The maximum Callus formation, root, and shoot number produced by 0.4 mg l^{-1} IAA with 4 mg l^{-1} VB1. High protoplast isolated in 0.5% sucrose. VB1 and Br enhanced the effect of IAA on root and shoot emergence of *Roses*.

Keywords: Callus, Phytohormone, Root, Shoot

Introduction

Damask rose (*Rosa damascena* Mill.) is one of the aromatic and medicinal plants whose essential oil has nutritional and economic values (Khosh-Khui, 2014). Cutting and grafting methods were used for *R. damascena* propagation (Badzhelova, 2017). The plant species is a valuable factor in inducing organogenesis by *in vitro* hormones (Jabbarzadeh and Khosh Khui, 2005). Regenerative, growth, cell, and tissue development studies during organogenesis *in vitro* culture can provide physiological, biochemical, and cellular information (Ginova *et al.*, 2012). Plant regulators conduct tissue differentiation to roots and shoot formation (Nak-Udom *et al.*, 2009). *In vitro* organogenesis study of three parts of plants including apical meristem, somatic embryo formation, and anatomy and histology studies to find differentiation and abnormalities (Ginova *et al.*, 2012). Contsary to the plant's origin from a zygote, *in vitro* plants originate from lateral buds of explants or somatic embryos. *In vitro* plant organogenesis depends on growth conditions, type of explants, and culture medium. The division of the phloem parenchymal cells forms the meristem site, which leads to the formation of the root and shoot pattern (Borkowska, 2001). Some studies have shown that thiamine is associated with cytokinin and has a role in inducing callus growth and rooting (Abrahamian and Kantharajah, 2011). *In vitro* culture of *R. damascena* shoot explants in medium containing 2000 mg l^{-1} indol butyric acid (IBA) resulted in rooting (Kafi-Falavarjani *et al.*, 2004). A combination of 2.5 mg l^{-1} BA and 0.1 mg l^{-1} IBA was the most suitable treatment for *R. damascena* propagation (Jabbarzadeh and Khosh-Khui, 2005). Hormones, as intracellular stimuli, act in a specific place and time, then they can change the direction of regeneration and development (Khosh-Khui, 2014). Thiamine (vitamin B1, VB1) is a factor for the enzymes involved in amino acids synthesis, tricarboxylic acid cycle, and pentose phosphate pathway. Vitamine B1 (thiamine) influenced the processes of rooting of plants (Goyer, 2010). Brassinosteroids (BRs) are common plant material structurally to animal steroid hormones that can function as plant growth regulators (phytohormones). Exogenous application of BRs effects including cell expansion, vascular differentiation, reproductive development, seed germination, multiple shoot induction, flowering, and fruit set (Haubrike and Assmann, 2006). Brassinosteroids which are natural compounds are known to stimulate stem elongation, control of apical dominance, and multiple shoot formations (Pereira-Netto *et al.*, 2003). VB1 has a high stimulating potential for root and shoot proliferation *in vitro* culture of plants (El-Mahdy and Yossef, 2019). We decided to investigate the effects of brassinosteroid and vitamin B1 on *in vitro* reproduction of *R. damascene* because we did not find any study on the effects of Brassinosteroids and Vitamin B1 on *in vitro*

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reproduction of Rosa.

This study aimed was to find the effects of IAA and 2, 4-D with Kin and BAP, thiamine (VB1), and brassinosteroid (Br) to increase rooting and shoot formation *in vitro* propagation of *Rosa damascena*. There was also a test for protoplast isolation by a simple protocol.

Methods and materials

Plant material: We bought the *Rosa damascena* plant (Isfahan cultivar 8:93) from Rose Research Center of Ahoora of Jahad Keshavarzi in Hamedan, Iran. Shoot explants with a node were used for *in vitro* culture, after disinfection with 5% hypochlorite for 15 minutes and washing with sterile water.

***In vitro* culture:** The nutrient culture medium consisted of MS salts (Murashige and Skoog, 1962), vitamins and organic compounds including Myo-inositol (100 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), pyridoxine- HCl (0.5 mg l^{-1}), thiamin-HCl (1 mg l^{-1}) and glycine (0.2 mg l^{-1}), 0.6% agar, and 3% sucrose. As plant growth regulators in this study, from 6- benzyl amino purine (BAP), 3- indole acetic acid (IAA), 2, 4-dichloro phenoxy acetic acid (2, 4-D), vitamin thiamine (VB1), and levonorgestrel brassinosteroid were used. We randomly evaluated the type and amount of hormones to compare brassinosteroid and vitamin effects on the organogenesis in the presence of cytokinins and auxins in two different tests. Plant regulators were used to prepare two test groups:

Test 1 (IAA and Kin with VB1 or Br): 0.1 and 1 mg l^{-1} IAA with 0.1 and 1 mg l^{-1} Kin, VB1 or Br (Table 2).

Test 2 (IAA, 2, 4-D and BAP with VB1 or Br): 0.4 and 4 mg l^{-1} IAA, BAP, 2, 4-D, VB1, and Br (Table 4). The medium pH was adjusted to 5.6 and then autoclaved at 1-atmosphere pressure and 121 °C for 15 minutes. Young- fresh shoot explants (1cm length) were cultured in MS medium. *R. damascena* explants *in vitro* culture data including rooting, callus, and shoot formation studied after fourth weeks.

Protoplasts isolation: Protoplasts were isolated from fresh young leaves. First, the leaves were disinfected with 5% hypochlorite, washed with sterile water, and chopped into very tiny pieces. The pieces placed in 2 ml of enzyme solution contained a semi-concentrated MS medium with sucrose (0, 0.5, 1, 1.5, and 2%) and 2% of each in the cellulose and pectinase (Merck) enzymes. After 2 to 4 hours, each sample was placed on a shaker for 5 minutes at 20 rpm. Then, a drop of solution was placed on the neobar slide, and was counted the isolated cells and protoplasts in each sucrose treatment. We found the best sucrose concentration for the isolation of healthy cells and protoplasts.

The experiments were conducted in ANOVA one away (all the experiments examined in at least three replication). Static analysis of data calculated by Minitab software and means were compared with Turkey's test ($P \leq 0.01$).

Results

Analysis of variance of the effect of IAA, Kin, VB1, and BR treatments with 0.1 and 1 binary and quaternary concentrations (8 treatments) showed that the difference between treatments in terms of rooting, shoot, and callus formation, was significant (Table 1).

As the findings of Table 2 show, T06 (1 mgl^{-1} IAA + 0.1 mgl^{-1} Br) had the highest rooting (63.33%) and T04 (1 mgl^{-1} IAA + 0.1 mgl^{-1} VB1) had the highest shoot formation (66.67%) and T02 (1 mgl^{-1} IAA + 0.1 mgl^{-1} Kin) had the highest callus production (19%). T08 (1 mgl^{-1} of all hormones) had the lowest rooting (8.33%), T07 (0.1 mgl^{-1} of all hormones) had the lowest shoot formation (0%). T01 (0.1 mgl^{-1} IAA + 1 mgl^{-1} Kin) and T07 (0.1 of all hormones) were free of callus formation. T03 (0.1 mgl^{-1} IAA + 1 mgl^{-1} VB1) was suitable for rooting (40%), shoot formation (50%) and low callus (10%) simultaneously (Figure 1).

Analysis of variance of the effects of IAA, BAP, 2, 4-D, VB1, and BRs in concentrations of 0.4 and 4 mg l^{-1} (12 treatments) on rooting, shoot and callus, and root and shoot numbers from test 2 experiment (Table 3) showed the difference between finding was significant.

According to the data in Table 4, rooting was observed in all treatments except T2 (0.4 mg l^{-1} IAA + 4 mg l^{-1} BAP). The highest rooting (73.33%) was observed in T3 (4 mg l^{-1} 2, 4-D + 0.4 mg l^{-1} BAP) which was almost equal to shoot formation (70%) but in which less callus formation happened (45%). Also, in T3 root number was low (1.8%) but the number of shoots was high (4.33%). The highest shoot (95%) in T7 (0.4 IAA + 4 BR) and the highest shoot with the highest callus (95%) were observed in T5 (0.4 mg l^{-1} IAA + 4 mg l^{-1} VB1) and T8 (4 mg l^{-1} IAA +0.4 mg l^{-1} BR). In T11 and T12, the average of the root number was low whereas the number of shoots was high. The highest root number (5.66%) and shoot number (5.33%) were observed in T5. T5 produced also the highest callus, shoot, and the high root. In T10-T12, the lowest root number (0.83%) was observed. In these treatments, the lowest rooting was with a low shoot and no callus formation. Also, T5 and T8 had good rooting (48.33% and 36.67%) with a high shoot and callus formation.

Protoplast isolation: As the data in figures 2, 3 show, at all concentrations of sucrose (S), after two hours, almost a large number of cells were isolated, there was no significant difference between them, but the number of protoplasts was very small. The differences in protoplasts between different concentrations of sucrose were significant. After 4 hours, the number of normal isolated cells was high and there was no significant difference, but the number of protoplasts increased. The treatment of S2 had the highest number of healthy protoplasts with 0.5% sucrose. In other treatments, the protoplasts were folded and compressed as sucrose increased. In S1, without sucrose, most protoplasts disintegrated.

Table 1. Comparison of analysis of variance of the effect of IAA, kinetin, VB1, and BR treatments on the shoot, root, and callus formation from shoot explants of *R. damascene*.

Source	DF	Mean square		
		Root	shoot	Callus
Treatments (T1-T8)	7	1337.3*	1518.7*	124.42*
Error	16	12.9	14.1	14.75
Total	23	-	-	-
CV%		48.41	50.90	56.31

***Significant in P<0.05**

Table 2. Mean comparison of the effect of IAA, Kin, VB1, and BR treatments (T01-T08) on root, shoot, and callus formation.

N.	Hormones mg l^{-1}				Root %	Shoot %	Callus %
	IAA	Kin	VB1	Br			
T01	0.1	1	-	-	11.00±1.00^{d}	23.33±2.89^{c}	0.00±0.00^{d}
T02	1	0.1	-	-	11.67±1.53^{d}	29.00±3.61^{c}	19.00±6.56^{a}
T03	0.1	-	1	-	40.00±5.00^{b}	50.00±5.00^{b}	10.00±5.00^{a}
T04	1	-	0.1	-	20.00±5.00^{c}	66.67±2.89^{a}	6.67±2.89^{b}
T05	0.1	-	-	1	48.33±2.89^{b}	01.67±2.89^{d}	1.67±2.89^{c}
T06	1	-	-	0.1	63.33±2.89^{a}	25.00±5.00^{c}	10.00±5.00^{a}
T07	0.1	0.1	0.1	0.1	10.00±5.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
T08	1	1	1	1	8.33±2.89^{d}	20.00±5.00^{c}	8.33±2.89^{a}

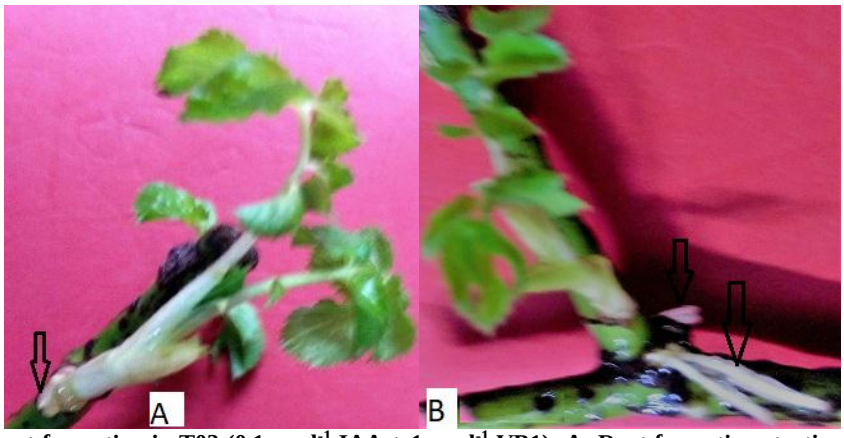

Figure 1. Shoot and root formation in T03 (0.1 mg l^{-1} IAA + 1 mg l^{-1} VB1): A- Root formation starting after third weeks. B- Root growth after fourth weak.

Discussion

BR effectively stimulated the elongation and formation of lateral shoots and shoot buds (Malabadi and Nataraja, 2007). VB1 is an essential element for *in vitro* propagation of *R. hybrid*. The concentration of 0.8 mg l^{-1} of VB1 had the highest growth rates of the *in vitro* propagated plantlets (Saad *et al*., 2016). Mixes of IBA and VB1 resulted in a higher number of roots and length roots on cuttings of azalea (Hou *et al*., 2020). The concentration of 0.4 mg l^{-1} IAA with 4 mg l^{-1} VB1 and 4 mg l^{-1} IAA with 0.4 mg l^{-1} BR with the high shoot and callus formation (48.33% and 36.67%) also had good rhizogenesis. *In vitro* effect of BR on multiple shoots was found to be the best at 1 mgl^{-1} with 3 mg l^{-1} BA, and root formation induced in BR alone (Verma *et al*., 2012).

The combination of low levels of IAA with high concentrations of VB1, and high concentrations of IAA with low BRs, in addition to stimulating shoot and callus formation, also stimulated rooting. Indole butyric acid (IBA) is an auxin commonly used for *in vitro* rooting and noted that other auxins such as NAA and IAA applied for root formation although there are less successful. The highest number of roots and shoots formation observed in T5. VB1 with IAA showed a positive effect on increasing the number of roots and multiple shoots. In MS medium containing BAP, multiple shoots and roots formed on damask rose explants (Badzhelova, 2017). The best results for rooting was related to 0.3 mg l^{-1} IBA with 0.3 mg l^{-1} NAA (Mirshahi *et al*., 2020). In MS medium, high IAA with low BAP (T1) also had rooting (28.33%), shoot formation (71.67%), and callus formation (70%) but was less than the maximum effect of some treatments. Appropriate concentrations of cytokinin and auxin together resulted in callus induction (Ginova *et al*., 2012). The results of this study showed that contrary to other reports, the presence of low levels of VB1 (0.1 mg

Table 3. Analysis of variance of the effect of IAA, BAP (instead of Kin), 2, 4-D, VB1 and BR treatments (T1-T12) on root, shoot and callus formation, root number, and shoot number.

Source	DF	Mean square				
		Root	Shoot	Callus	Root Number	Shoot Number
Treatments (T1-T12)	11	1455.1*	3005*	4229.5*	7.9647*	9.10*
Error	24	29.9	36.8	14.6	0.1656	0.94
Total	35	-	-	-	-	-
CV%	-	49.59	28.64	62.01	90.55	33.07

***Significant in P<0.05**

Table 4. Mean comparison of the effect of IAA, BAP, 2, 4-D, VB1, and BR treatments (T1-T12) on root (R), shoot (Sh), and callus (C) formation, root number (RN), and shoot number (ShN).

N.	Hormones mg l^{-1}		R%	Sh%	C%	RN%	ShN%
T1	IAA 4	BAP 0.2	28.33±2.89^{c}	71.67±5.77^{b}	70.00±5.00^{b}	0.38±0.20^{c}	4.17±0.29^{a}
T2	IAA0.2	BAP4	0.00±0.00^{e}	53.33±10.41^{b}	46.67±2.89^{c}	3.50±0.20^{a}	1.67±1.15b^{a}
T3	2,4-D4	BAP 0.2	73.33±2.89^{a}	70.00±5.00^{b}	45.00±5.00^{c}	1.80±0.70^{ab}	4.33±0.58^{a}
T4	2,4-D0.2	BAP4	10.00±5.00^{d}	66.67±2.89^{b}	70.00±5.00^{b}	5.65±0.65^{a}	5.00±1.00^{a}
T5	IAA0.2	VB14	48.33±11.55^{b}	95.00±5.00^{a}	95.00±5.00^{a}	1.53±0.68^{b}	5.33±1.53^{a}
T6	IAA 4	VB10.2	10.00±5.00^{d}	13.33±2.89^{c}	6.67±2.89^{d}	0.32±0.02^{c}	1.17±0.76^{b}
T7	IAA0.2	Br4	21.67±2.89^{c}	95.00±5.00^{a}	2.00±5.00^{d}	1.17±0.15^{b}	2.33±1.53^{ab}
T8	IAA 4	Br0.2	36.67±2.89^{c}	95.33±2.89^{a}	95.00±5.00^{a}	0.18±0.57^{c}	3.67±2.08^{ab}
T9	VB14	BAP 0.2	25.00±10.00^{c}	23.33±10.41^{c}	3.33±2.89^{d}	0.26±0.16^{c}	1.50±0.87^{ba}
T10	VB10.2	BAP4	10.00±5.00^{d}	16.67±7.64^{c}	0.00±0.00^{d}	1.25±0.66^{ab}	0.83±0.29^{b}
T11	Br4	BAP 0.2	1.67±2.89^{e}	30.00±5.00^{c}	0.00±0.00^{d}	0.92±0.14^{c}	0.83±0.29^{b}
T12	Br0.2	BAP4	1.67±2.89^{e}	33.33±2.89^{c}	0.00±0.00^{d}	0.10±0.10^{cd}	0.83±0.29^{b}

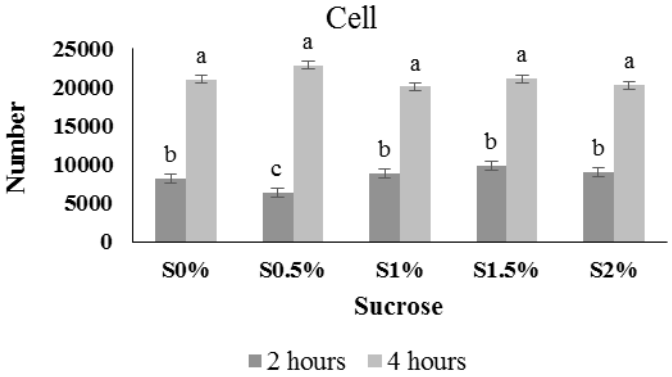

Figure 2. Cell number in different concentrations of sucrose in enzymatic medium, after 2 and 4 hours.

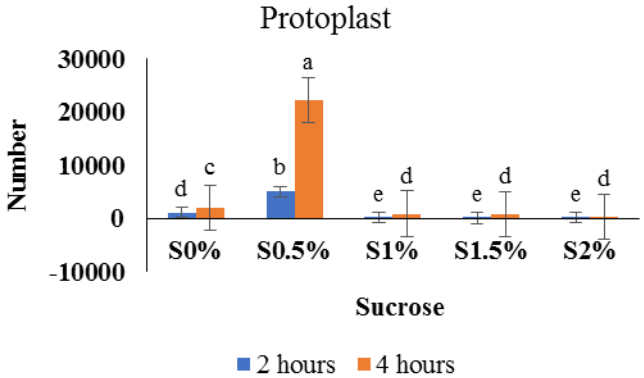

Figure 3. Protoplast number in different concentrations of sucrose in enzymatic medium, after 2 and 4 hours.

l^{-1}) with IAA stimulates shoot morphogenesis, and with low levels of BRs (0.1 mg l^{-1}) with IAA (1mg l^{-1}) stimulate rooting. For simultaneous root and shoot formation without subculture, a low amount of IAA (0.1

mg l^{-1}) with a higher VB1 (1 mg l^{-1}) was suitable. The highest shoots on the explants of damask rose observed when shooting explants kept in the induction medium (1/2 MS + 3% sucrose + 6.8 µM thidizuron + 0.27 µM NAA) for three weeks, and later transfer to regeneration medium (MS + 2.25 µM BA + 0.054 µM NAA). Histological studies revealed the direct formation of shoot buds without the intervening callus phase (Kumar-Pati *et al*., 2004). The highest shoot formation of *R. damascene* explants have obtained in the medium with 0.6% agar and 4 mg l^{-1} benzyl adenine (BA) (Doina *et al*., 2017).

In general, callus formation in both levels of IAA with both Kin levels, VB1, Br, and altogether was very low. These results showed that in these treatments, rooting and shoot formation are completed directly without callus formation. The concentration of 0.5 mg l^{-1} VB1 and 0.2 mg l^{-1} biotin was the optimum concentration for giving the highest number of shoots and somatic embryos of date palm (Al- Khayri, 2001). Our findings showed that 2, 4-D with BAP is suitable for rooting, shoot, and callus formation, especially rooting. This treatment was better for rooting (a problem *in vitro* propagation of damask rose) than other treatments. Shoot explants of damask rose produced multiple shoots in MS medium containing 0.1 mg l^{-1} IAA or 4 mg l^{-1} 2, 4-D accompanied by a cytokinin (Khosh-Khui, 2014). Callus induction is not a desirable trait but observed due to the presence of auxins in the culture medium (Ginova *et al*., 2012). Kin and BAP alone or with auxins are drastic for shoot formation in some woody plants (Jabbarzade and Khosh Khui, 2005). Very little researches on the isolation and culture of protoplast from *R. damascena* access have been reported (Ginova *et al*., 2012). This finding is consistent with the reports of Kumar-Pati *et al*. (2004), which isolated protoplasts from *Rose* species with sucrose for osmotic pressure. We accessed a large number of cells and a low number of protoplasts from tiny pieces of leaves in a sucrose-containing medium with cellulase and pectinase after two hours. We obtained the highest number of protoplasts in 0.5% sucrose, after 4 hours. In our protocol, time was less, speed and efficiency were higher. This protocol can be a quick procedure for protoplast isolation from damask rose.

The results showed that thiamine (vitamin VB1) and brassinosteroid (Br) enhance the effect of indole acetic acid (IAA) on the rooting and shoot emergence of roses. The half force of MS salts with 2% cellulase, 2% pectinase, and 0.5% sucrose were a simple protocol for protoplast isolation from damask rose leaves. This protocol proposes a new method for isolating protoplast from damask rose leaves.

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