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Research Article

In vito organogenesis origin site detection with explants anatomical study of damask rose (*Rosa damascena* Mill.)

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Abstract

***Damask rose is* one of the aromatic and medicinal plants belonging to the Rosacea family whose essential oil has nutritional and economic value. The purpose of this research was to determine the location of the hormones effects in organ tissues for the formation of calluses, buds and roots. For this, we have studied hormone effects on the callus, shoot, root formation, and anatomical alternate. We found IAA and 2, 4-D with BAP were affected by callus, shoot, and root production from damask shoot explants. Regeneration organs were undifferentiated to callus after two weeks. To avoid this problem, we suggest that it is better to subculture explants in a new medium with fewer hormones. The site of callus, shoot, and root formation was the node on the explant. This explant node had a lateral meristem. The anatomical study of explants showed that the origin of callus, shoot, and root formation is cambium parenchymal cells.**

Keywords: Cytokinin, Auxin, Shoot, Root, Cambium, Meristem

Introduction

Damask rose (*Rosa damascena* Mill.) is one of the aromatic and medicinal plants whose essential oil has nutritional and economic value (Khosh-Khui, 2014). The combination application of appropriate light intensity and mulch could be considered for sustaining soil fertility and enhancing damask rose crop productivity (Takure and Kumar, 2021). Plant species and their tissues are the main factors in inducing organogenesis by in vitro hormones (Jabbarzadeh and Khosh-Khui, 2005). Cytokinin and auxin in equal concentration together result in callus induction (Ginova *et al*., 2012). Damask rose shoot explants 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 (Masomboon and Boomoomiri, 2003). Damask rose explants grew and formed shoots in low light. Damask rose explants rooting increased in dark conditions (Khosh-Khui and Sink, 1982). Callus induction is not a desirable trait. Calogenesis induced due to the presence of auxins in vitro culture (Ginova *et al*., 2012). Kinetin and BAP alone or in combination activated the shoot formation of some woody plants (Jabbarzadeh and Khosh-Khui, 2005). Indole butyric acid (IBA) is one of the auxins commonly used for in vitro rooting. Other auxins such as NAA and IAA, are utilized for root regeneration, but they are less successful. Hormones, as intracellular stimuli, act at a characterized place and time, and they modify regeneration and development in various ways (Nikbakht *et al*., 2005). Detection of plant regeneration and growth transformation (during in vitro organogenesis) provides physiological, biochemical, and cellular events information for organogenesis orientation. (Ginova *et al*., 2012). Contrary to the plants produced from the zygote, in vitro plants originate from the lateral buds of explants or somatic embryos, depending on growth conditions, type of explants, and culture medium. The main factor in transferring the plant from the glass to the pot and its survival is the vascular connection of the shoots to the roots (Borkowska, 2001). In vitro organogenesis studies consist of three parts: 1- apical meristems and their development to the roots or shoots. 2- Development of the somatic embryo and 3- Anatomical and histological study to find changes and abnormalities events (Ginova *et al*., 2012). Hormones can determine tissue differentiation ways; and root and shoot production (Nak-Udom *et al*., 2009). The initial primordia of the root are characterized by the xylem, phloem, and pith tissue building. The division of the phloem

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parenchymatic cells produces meristematic tissue. Meristematic tissue makes the root primordia (Borkowska, 2001). The purpose of this research was to determine the location of the hormones effects in organ tissues for the formation of calluses, buds and roots. For this, we have studied hormone effects on the callus, shoot, root formation, and anatomical alternate.

Methods and materials

Damask rose, Clone Isfahan 8, No. 93 was prepared by the Hamedan Research Institute of Iran. Young shoot explants were cultured in MS medium after disinfection with 5% hypochlorite for 15 minutes and washed with sterile water. The nutrient culture medium consisted of MS salts and vitamins with 0.8% agar and 3% sucrose (Murashige and Skoog, 1962). MS medium with a combination of 0.2 and 4 mg ml^{-1} BAP (6-benzyl amino purine), IAA (3-indole acetic acid), and 2, 4-D (2, 4-dichloro phenoxy acetic acid) to prepare 4 Treatment (T1-T4) was used (Table 2) (Masomboon and Bhoomsiri, 2003). The medium pH was adjusted to 5.6 and then autoclaved at 1-atmosphere pressure and 121°C for 15 min.

To study the effects of treatments on explant anatomy, explants were removed from the culture medium after 0 to 4 weeks and placed in the FAA fixator (70% ethanol: glacial acetic acid: formalin; 90: 5: 5). After 2, 3, and 4 weeks, changes in appearance were observed. Cross-sections prepared from the samples were decolorized with 5% hypochlorite (10 min) and stained with 1% methylene blue (1 min) and alum carmine (20 min) (Mehrabian *et al*., 2007). The sections were studied using a ZEISS optical microscope with a magnification of 100- 400.

The experiments were performed in a completely randomized design (experiments were repeated three times). All the treatments were statistically analyzed using Minitab software, and the mean comparisons were analyzed with Turkey's test ($P \leq 0.05$).

Results

Analysis of variance showed that the differences between treatments in terms of callus, shoot, rooting, and necrosis concerning time, treatment, and the interaction of the two factors were significant (Table 1). The data in Table 2 show that T1 in the third week and T4 in the second week, without significant difference, had the highest callus. T2 and T3 had the lowest calluses. Shoot formation was the highest in T1 after the third week and in T4 after the second week. New shoots regenerated in most treatments. In all treatments except T2, rooting has started in the second and third weeks. Rooting in T3 in the third week was more effective than other treatments. In all treatments, in the third or fourth week, callus production and shoot and root regeneration decreased because of explants necrosis.

Before culture (at zero time), anatomical studies of shoot explants (Figure 1) showed that the epidermis contains a cellular layer. The hypodermic layer is below it. Hypodermis consists of multicellular bodies with 1-3 cell layers, some of which originated from cork cambium. The multi-collenchyma bodies were dissimilar in width and length, with 1-3 and 3–35 cells, respectively. The thickness of the cortex varied on different sides of the shoot and contained 10–14 cells. Secretory canals and calcium carbonate storage cells were in the cortex tissue. In the vascular cylinder, there was a cambium with 3–4 cells, secondary xylem toward the interior, and secondary phloem toward the exterior. The secondary xylem and phloem were in the form of separate bodies. The number of vascular bundles was high and ranged from 30 to 37. The number of sclerenchyma caps observed in the cortex was equal to those of primary xylem and phloem bundles. The xylem and phloem bundles were different in size. The size of sclerenchyma caps varied depending on the size of xylem and phloem bundles. In most bundles, the primary xylem and phloem necrosed with the formation of the secondary tissue. In one or two parts of the vascular cylinder, 1–4 primary phloem bundles remained and were visible as a mass different from the other bundles. These bodies were the sites of meristematic cells formation. The explant pith contained parenchyma, sclerenchyma, and secretory canals.

The analysis of the explants anatomy revealed that hormonal treatments were active on the formation and number of cells of some tissues. According to the statistical data in Table 3, the effects of W and T and the W/T have significant differences in meristematic bodies, the number of cork cambium bodies, and the pericycle layer ($P \leq 0.05$). The effect of time was also significant ($P \leq 0.05$) on the vascular cambium.

The anatomical study of explants in the second, third and fourth weeks indicated that meristematic bodies were formed, swelled, and removed from the vascular cylinder during the 2–4 weeks with the typical division of cambium cells. Meristematic bodies increased in T1 in the 4th week after culture (Figure 2A). In treatment 1 (T1), the number of meristematic bodies was higher than in the other treatments (Table 4). After T1, the number of meristematic bodies was high in T4. Cell number of vascular cambium bodies was similar, with no significant difference in all treatments, except for the fourth week of T1. The number of cambium cells decreased sharply in the 4th week of T1. The number of cork cambium cells in the 4th week of T1 peaked. Cell number of cork cambium was less than a significant difference in T3 and T4 (Table 4). The number of cells in the cortex width did not change, but they pressed.

Collenchyma cell layers decreased to a 0–1 cell layer (Figure 2B). From 2 to 3 weeks, primary vascular bundles pushed inward to the central cylinder. The vessels were squeezed and crushed, with increasing cell number and the effect of the hormone on vascular cambium cells. The cortex and the epidermis tissue fissioned, and the callus appeared as undifferentiated tissue by increasing cell number (Figures 2C, D). Massive calluses were produced at the surface of the

Table 1. Analysis of variance for time (W), treatments (T1–T4), and W/T effects on callus induction, shoot and root formation, and explants necrosis.

SOV	DF	Mean square			
		Callus	Shoot	Root	Necrosis
W	2	0.0756ns	0.0200*	0.014*	0.0137*
T	3	0.0672*	0.0178*	0.0238*	0.0122*
W/T	11	0.0550*	0.0146*	0.0138*	0.0100*
Error	23	0.0416	0.011	0.0109	0.0075

SOV: source of variation; ns: not significant; *: significant difference at P ≤ 0.05 (Tukey's test).

Table 2. Callus induction, shoot formation, rooting, and necrosis of explants in T1-T4 at 2-4 weeks (W2-W4).

N.	Growth Regulator mg L^{-1}	Callus %	Shoot %	Root %	Necrosis %
T1W2	0.2 BAP, 4 IAA	60^{b}	35^{d}	0^{g}	0^{g}
T1W3	0.2 BAP, 4 IAA	100^{a}	100^{a}	0^{g}	35^{d}
T1W4	0.2 BAP, 4 IAA	70^{b}	75^{ab}	30^{de}	35^{d}
T2W2	4 BAP, 0.2 IAA	5^{f}	5^{f}	0^{g}	0^{g}
T2W3	4 BAP, 0.2 IAA	5^{f}	15^{e}	0^{g}	0^{g}
T2W4	4 BAP, 0.2 IAA	50^{c}	60^{b}	0^{g}	0^{g}
T3W2	0.2 BAP, 4 2,4-D	30	100^{a}	30	0^{g}
T3W3	0.2 BAP, 4 2,4-D	45^{cd}	70^{b}	70^{b}	30^{de}
T3W4	0.2 BAP, 4 2,4-D	0^{g}	0^{g}	0^{g}	100^{a}
T4W2	4 BAP, 0.2 2,4-D	100^{a}	100^{a}	35^{d}	0^{g}
T4W3	4 BAP, 0.2 2,4-D	70^{b}	70^{b}	15^{e}	30^{de}
T4W4	4 BAP, 0.2 2,4-D	30^{de}	30^{de}	15^{e}	70^{b}

Means with a different letter in a column are statistically differents (Tukey's test, P ≤ 0.05).

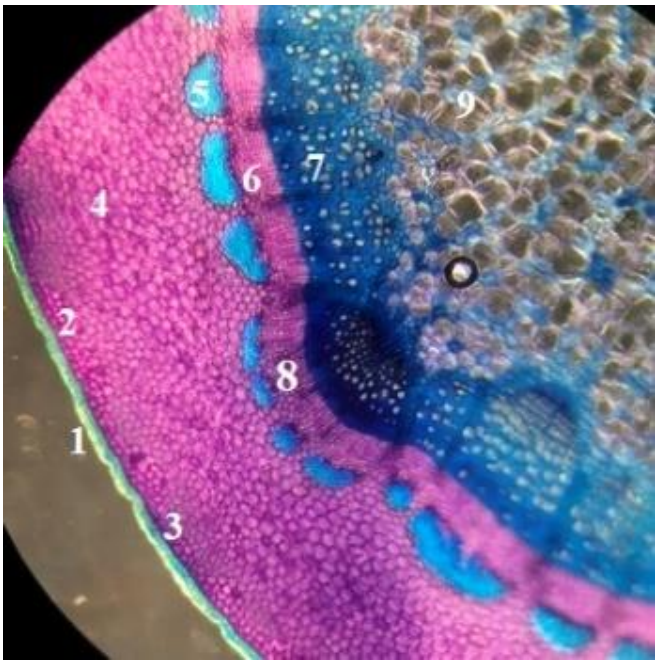

Figure 1. Anatomy of shoot explant at zero time in vitro: 1. Epidermis, 2. Collenchyma hypodermis, 3. Phellogen, 4. Cortex, 5. Sclerenchyma cap, 6. Secondary phloem, 7. Secondary xylem, 8. meristematic bodies, and 9. Pith.

Table 3. Analysis of variance for the effects of time (W), treatment (T), and W/T on meristematic bodies (MA), pericycle (P), number of vascular cambium (NVC), cork cambium cells (PC), vessel group (VGN), xylem vessel (XVN), collenchyma cell (ClCN), and cortex cell (CCN) in shoot explants (in vitro culture).

SOV	F	Mean square							
		MA	NVC	PC	P	VGN	XVN	ClCN	CCN
W	2	1.6475^{*}	2.3332^{*}	1.5883^{*}	0.2500^{*}	11.2934^{ns}	17.7527^{*}	28.0408^{*}	13.4213^{ns}
T	3	1.6192^{*}	2.0740^{ns}	1.4071^{*}	0.2222^{*}	9.0530^{ns}	15.7802^{ns}	24.2585^{*}	11.9274^{ns}
W/T	11	2.3418^{*}	1.5151^{ns}	1.1512^{*}	0.1818^{*}	7.4970^{ns}	12.9175^{ns}	19.8478^{*}	9.7588^{ns}
Error	23	1.4744	1.1979	0.8722	01376	5.2764	9.7799	15.0949	7.390

SOV: source of variation; F: Degree of Freedom, ns: not significant; *: significant at P ≤ 0.05 (Tukey's test).

Table 4. Comparison of the mean effect of treatment (T) and time (W) on the formation of meristematic bodies (MA), pericycle (P), number of vascular cambium (NVC), cork cambium cells (PC), vessel group (VGN), xylem vessel (XVN), collenchyma cell (ClCN), and cortex cell (CCN) in shoot explants.

T	P	MA	PC	VC	VGN	VN	ClCN.	CCN.
T0W0	0^{e}	0.3^{e}	4^{a}	4^{a}	36^{a}	6.7^{d}	30^{b}	10^{d}
T1W2	0.3^{c}	3.3^{b}	0.3^{d}	3.7^{ab}	32^{b}	8.7^{b}	12^{d}	12.7^{c}
T1W3	1.3^{b}	3.7^{b}	0^{e}	3.7^{ab}	26^{d}	8.7^{b}	12^{d}	12^{c}
T1W4	2.7^{a}	6.3^{a}	4^{a}	1.7^{c}	21^{ef}	9.3^{ab}	25^{c}	8.67^{e}
T2W2	0^{e}	1.3^{d}	0^{e}	3.3^{b}	32^{b}	6.7^{d}	8.3^{e}	20.3^{a}
T2W3	0^{e}	0.3^{e}	0^{e}	3.3^{b}	20^{f}	11^{a}	30^{b}	12^{c}
T2W4	0^{e}	0.3^{e}	0.3^{d}	4^{a}	30^{c}	5^{e}	30^{b}	12^{c}
T3W2	0^{e}	0^{f}	0.3^{d}	4^{a}	35^{a}	5.3^{e}	30^{b}	11.7^{cd}
T3W3	0^{e}	3^{bc}	1^{c}	4^{a}	30^{c}	7.7^{c}	31^{ab}	10^{d}
T3W4	0^{e}	0.3^{e}	1^{c}	4^{a}	30^{c}	7.7^{c}	31^{ab}	10^{d}
T4W2	0^{e}	3^{bc}	2.3^{b}	4^{a}	22^{e}	10^{a}	14^{d}	12.7^{c}
T4W3	0^{e}	2^{c}	0.7^{cd}	4^{a}	34^{b}	10^{a}	32^{a}	14.7^{b}
T4W4	0^{e}	3.3^{b}	0.7^{cd}	4^{a}	34^{b}	10^{a}	32^{a}	14.7^{b}

Means with a different letter in a column indicate a significant difference (Tukey's test, P≤ 0.05).

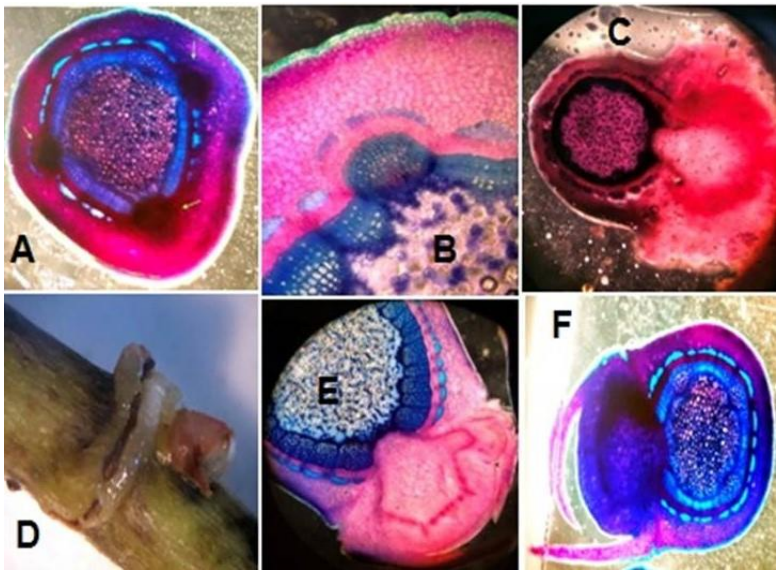

Figure 2. A. Increase in the number of meristematic bodies including primary xylem and phloem bundles in T1 during the second week, B. Changes in vascular bundles and surrounding cells as swelling into the cortex and formation of meristematic bodies in T1 during the 2nd week; C. Emergence of undifferentiated cell bodies as callus from the meristematic bodies formation site in T1 after 4 weeks; D. Appearance of callus induction in T1 after 4 weeks; E. Formation of leaf shape and shoot apical meristem without differentiation of cellular regions and callus tissue formation on both sides in T4 in the 4th week; F. Callus induction from growth and division of one meristematic.

explant in the 4th week. The shoot formation was similar to that of callus except for the primary structure of vessels and bush leaves formed, representing cell differentiation (Figure 2E). BAP and IAA acted on cambium tissue to meristematic bodies, shoots, and non-differentiated callus cells. Cellular changes for T2, T3, and T4 were similar to callus induction treatments (Figure 2 F). There weren't any changes in the root formation in the pericycle layer. The number of collenchyma bodies located below the epidermis (Table 4) and the effects of W, T, and W/T were significant (P≤ 0.05). The effect of time on the number of xylem bundles change in each vessel group was significant.

Discussion

Damask shoot explants in MS medium with concentrations of 0.2 and 4 mg L^{-1} IAA and 2, 4-D, along with 0.2 and 4 mg L^{-1} BAP which resulted in callus production, shoot formation and rooting. However, growth stopped with necrosis, and the explants were destroyed in the 3rd and 4th weeks. These results are consistent with the fact that cytokinins and auxins can determine tissue differentiation and morphogenesis for root and shoot formation. In vitro, roots can be formed directly from plant tissues or calluses (Nak-Udom *et al*., 2009). Appropriate concentrations of cytokinin and auxin together result in callus induction, so auxin at high concentrations causes callus induction (Ginova *et al*., 2012). Indole butyric acid (IBA) is one of the auxins commonly used for in vitro rooting. It should be noted that other auxins such as NAA and IAA, are also used for rooting but are less successful (Nak-Udom *et al*., 2009). Based on the results of this study, rooting was performed on Damask

rose explants with 2, 4-D, and IAA along with BAP. However, the effect of these two hormones on rooting was small, the roots were short-lived, and after a while, they developed calluses or necrosis. Callus induction is not a desirable trait but occurs due to the presence of auxins in the culture medium (Ginova *et al*., 2012). The use of kinetin and BAP alone or in combination with auxins is effective for shoot formation in some woody plants (Jabbarzadeh and Khosh- Khui, 2005). There are some reports that BAP has negative effects on shoot propagation (Borkowska, 2001). In low light, the shoots produce and grow, but in the dark, the rooting of rose cultivars increases (Khosh-Khuei and Sink, 1982). In this study, by placing all specimens in the dark, callus production, shoot formation (100%), and rooting (up to 70%) were observed in all treatments, especially in T3 (4 mg L^{-1}, 2, 4-D) with 0.2 BAP. This result indicates that darkness is suitable for inducing calluses, shoots, and roots, but the duration should not be more than two weeks. Callus, shoot, and root regenerated on the buds of explants. Callogenesis and organogenesis depend on the type and amount of hormone, tissue location in organs, and plant species (Jabbarzadeh and Khoshkhoi, 2005). Hormones, as intracellular stimuli, act at a characterized place and time. They can change the ways of regeneration and development (Nikbakht *et al*., 2005). Tiamine and brasinostroids enhanced the effect of IAA on the root and shoot emergence of roses (Razaji *et al*., 2021). The present study indicated that in four treatments, explants developed necrosis and were destroyed after three weeks, suggesting that they should be subcultured in the new culture medium. Auxins and cytokinin acted on the vascular parenchymal tissue in the cambium. They prevented the establishment of vascular cambium, shoot, and root meristems. This result is similar to the report that divides the phloem parenchymal cells of the meristematic site and leads to the shoot or root patterns. In woody plants, the root meristem is produced from parenchymal cells outside the vascular cambium or cells of the cortical region (Borkowska, 2001). The shoot formation was similar to that of callus, except for the primary structure of vessels and bush leaves formed. The two hormones, BAP and IAA, were effective at the site of cambium tissue for meristem cells formation and the formation of shoots or non-differentiated callus cells. In this research, we determined that the callus and organogenesis site in explants of rose damask is cambium cells, which we have not found a report for so far.

Conclusion

In this study, we found that IAA and 2, 4-D with BAP are effective in callus, shoot, and root formation in damask rose, but the differentiation returns to callus formation after two weeks. To avoid this problem, we suggest that it is better to subculture explants in a new medium with fewer hormones. The location of callus, shoot, and root formation was the lateral bud with a lateral meristem. Cambium cells in stem explants were the site of hormone action for the induction of callus, bud and root in vitro culture. These results show that organogenesis can be achieved earlier and with less hormone in damask rose by separating the cambium tissue and cultivating it in an in vitro environment.

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