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Callogenesis and production of anthocyanin and chlorophyll in callus cultures of vegetative and floral explants in *Rosa gallica* and *Rosa hybrida* (Rosaceae)

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Abstract: Callogenesis using vegetative (leaf, stem, petiole) and floral (petal, pistil, anther) explants of *Rosa gallica* and *R. hybrida* was investigated using different combinations of 2,4-dichlorophenoxyacetic acid, 6-benzylaminopurine, and gibberellic acid on modified Murashige and Skoog medium. The highest callogenesis was obtained on Murashige and Skoog medium containing ratios of 2 and 3 mg L^{-1} of 2,4-dichlorophenoxyacetic acid to 1 mg L^{-1} of 6-benzylamino purine in both species depending on type of explant. Stem explants in *R. gallica* initiated callus after 4 days while the other explants in *R. gallica* and all explants in *R. hybrida* showed the greatest percentage of callus initiation after 8 days. The callus growth rate showed much more progressive callus growth respectively on leaf and stem explants in both species. The highest callus volume was achieved after 2 months from vegetative explants and after 2.5 months from flower explants in both species. The highest anthocyanin and chlorophyll yield was produced in vegetative calluses of *R. gallica* but their content was less in flower calluses. In *R. hybrida*, the highest value of pigment was observed in calluses from leaf and stem. It is noteworthy that anthocyanin content in different calluses, especially vegetative ones in *R. gallica*, was much higher, while chlorophyll concentration was somewhat more in calluses derived from explants of *R. hybrida*.

Key words: Calluses, explant, *Rosa gallica* var. *officinalis*, *Rosa hybrida* 'Dolcvita'

1. Introduction

Rose is one of the most important commercial flower crops used as ornamental plants and in the cut flower industry. Roses are popularly used in the perfume and cosmetic industries and for medicinal purposes in many regions of the world, such as *Rosa hybrida* L. (Esselink et al., 2003; Nadeem et al., 2013) and *Rosa gallica* L. (Ochir et al., 2010).

In the last few years, in vitro propagation has revolutionized commercial nursery business (Pati et al., 2006) with unique advantages, such as rapid multiplication of valuable genotypes, production of disease-free plants, and nonseasonal production throughout the year (Shabbir et al., 2009). Therefore, a tissue culture system in roses has been established (Ibrahim and Debergh, 2001; Kim et al., 2003; Hameed et al., 2006; Rout et al., 2006; Drefahl et al., 2007; Previati et al., 2008).

The genus *Rosa* has been the subject of numerous studies involving the in vitro culture of axillary buds, shoot tips, anthers, and embryos (Tabaeezadeh and Khosh-Khui, 1981; Burger et al., 1990; Pati et al., 2004; Vu et al., 2006). A cytokinin (usually 6-benzylamino purine [BAP]) and an auxin (mostly indole-3-acetic acid [IAA], 1-naphthalene acetic acid [NAA], or 2,4-dichlorophenoxyacetic acid [2,4-D]) are normally included in the primary culture medium for callus induction (Gürel et al., 2001; Chalager and Venkanna, 2012).

Genotypic variation has also been reported by different researchers by the use of growth regulators (type and combination) in callus induction of vegetative segments of rose (Hill, 1967; Lloyd et al., 1988; Rout et al., 1992; Hsia and Korban, 1996; Luciani et al., 2006; Ram et al., 2011), but little has been known about callogenesis of flower explants exposed to growth regulators. Therefore, one of the objectives of this study was callus initiation by the means of tissue culture and its comparison from 2 points of view, including different growth regulators and various explants, both vegetative and generative, in *Rosa hybrida* and *Rosa gallica* to assess the optimized callogenesis.

There is much research that has been done in vivo on anthocyanin and chlorophyll assessment for their vital roles (Karageorgou and Manetas, 2006; Liakopoulos et al., 2006; Terzi and Kadioglu, 2006; Gitelson and Memelink, 2009). Anthocyanins represent the major red, purple, violet, and blue pigments in many flowers and fruits. They attract pollinators and seed dispersers and defend plants

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against abiotic and biotic stresses (Petroni and Tonelli, 2011). Research on anthocyanin by plant cell and tissue cultures is increasing to find an effective system, such as food biotechnology (Koda et al., 1992; Nawa and Ohtani, 1992) or some disease treatments including anticancer and cardioprotective bioassays (Jackman et al., 1987; Wang and Jiao, 2000; Gantet and Memelink, 2002; Hou, 2003; Lila, 2004). Pigment recovery from the fresh materials involves such limitations as variability and seasonal availability of raw materials, fresh material losses, and pigment degradation caused by storage and the extraction process. This method has the added advantage of producing substances that are difficult to synthesize by alternative chemical methods.

There have been many other studies on the production of anthocyanin by cultured cells, such as sweet potato (Nozue et al., 1987), Rabbiteye blueberry (Nawa et al., 1993), and rose (Ram et al., 2011). For these reasons, the other aim of this study was the establishment of anthocyanin content in *Rosa gallica* and *R. hybrida* calluses produced on different explants, comparing its value in vegetative and generative segments for the first time. Another aim was to assess whether calluses derived from generative explants produce more anthocyanin, referring to their high concentrations responsible for coloration in reproductive organs in vivo.

Chlorophyll-containing tissues in culture would be useful for research on autotrophic growth, metabolism, and disease (Hildebrandt et al., 1963). Chlorophyll is the other factor that is measured in this study with the same interests as mentioned for anthocyanin content, but opposite from that to investigate if vegetatively derived calluses contain more chlorophyll due to the high concentration in vegetative parts in vivo.

2. Materials and methods

2.1. Plant material and establishment of culture

Vegetative (leaf, stem, and petiole) and floral (petal, anther, and pistil) explants were collected from *Rosa gallica* var. *officinalis* and *Rosa hybrida* 'Dolcvita' during the spring/summer season from plants of 3–4 years old. Healthy explants were surface-sterilized by washing in detergent for 10 min, the kept under tap water for 30 min, dipped in 70% ethanol for 1 min, immersed in 10% sodium hypochlorite plus 1% Tween 20 for 3 min, and embedded in Hg_2Cl_2 plus 1% Tween 20 for 3 min, followed by 3 rinses in sterile distilled water. Vegetative explants were cut into sections of about 8–12 mm and whole floral organs were used as explants for callus induction. The last stage was embedding in antibiotics, 10 mg L^{-1} ampicillin and 10 mg L^{-1} tetracycline, for 20 min each. Explants were inoculated horizontally on the surface of 30 mL of callogenesis medium in 9-cm petri dishes. The medium consisted of Murashige and Skoog (Murashige and Skoog, 1962) basal salts and vitamins supplemented with different concentrations of growth regulators including combinations of 2,4-D, BAP, and gibberellic acid (GA_3), sucrose (30 g L^{-1}), and agar (8.0 g L^{-1}). The pH was adjusted to 5.7–5.8 prior to autoclaving. All media were sterilized by autoclaving for 20 min at 121 °C (1.5 kg cm^{-2} pressure). Cultures were maintained in a 16-h photoperiod (2000 lx white cool fluorescence) at 23 ± 2 °C. Calluses were then routinely subcultured onto medium of the same composition at 2-week intervals.

2.2. Assay for pigment extraction

Anthocyanin and chlorophyll content were measured in calluses obtained from 14-, 30-, 45-, and 60-day-old different explants in each species. The comparison of pigment content between 2 species was carried out in 60-day-old calluses. The method of Mori et al. (1993) was applied in order to assess anthocyanin content of various calluses under dark and light conditions. Anthocyanin compounds were extracted from 0.1 g of fresh callus, ground in 10 mL of acidic methanolic solution (ratio of MeOH to HCl 99:1 V/V). The solution was kept under dark conditions for 24 h at 25 °C. The samples were centrifuged for 10 min at 4000 rpm and the supernatant was analyzed with a spectrophotometer at 550 nm. Anthocyanin content was calculated using the extinction coefficient (ϵ) of 33,000 cm^{-1} mol^{-1} and based on the following formula:

$$A = \epsilon bc,$$

where absorbance = A, cell width = 1 cm = b, and anthocyanin concentration = c. Total anthocyanin content was determined as μmol g^{-1} fresh weight (FW).

Chlorophyll analysis was performed by the Lichtenthaler (1987) method using various calluses under dark and light conditions. Fresh callus of 0.1 g was ground in 15 mL of 80% acetone. After straining, the absorption was analyzed with a spectrophotometer at 646.8, 663.2, and 470 nm. Chlorophyll content was calculated based on the following formulas:

$$Chla = (12.25A_{663.2} - 2.79A_{646.8}),$$

$$Chlb = (21.21A_{646.8} - 5.1A_{663.2}),$$

$$ChlT = Chla + Chlb,$$

where Chla = chlorophyll a concentration, Chlb = chlorophyll b concentration, and ChlT = total chlorophyll concentration. Final results were expressed as mg g^{-1} FW.

2.3. Statistical analysis

All experiments were carried out in a completely randomized design with 3 replicates. Data were statistically analyzed for significance by analysis of variance with mean separation by Duncan's multiple range test and means were separated using an error rate at the 5% level of significance with SPSS 9.

3. Results

The effect of plant growth regulators on callus formation showed that in *Rosa gallica* L., 2 mg L^{-1} of 2,4-D and 1 mg L^{-1} of BAP were the optimum concentrations, increasing callogenesis more than 90% in vegetative (leaf, stem and petiole) and petal explants and more than 80% in flower explants (pistil and anther) (Figure 1). In addition, 3 mg L^{-1} of 2,4-D and 1 mg L^{-1} BAP were suitable for callus induction (>90%) in pistils and anthers (Figure 1). Similarly, in *R. hybrida*, 2 mg L^{-1} of 2,4-D and 1 mg L^{-1} BAP was the best treatment for callogenesis of stem, petiole, leaf, and petal explants to more than 90%, while in pistil and anther explants, the 3 mg L^{-1} preparation of 2,4-D and 1 mg L^{-1} of BAP increased callogenesis by more than 85% (Figure 1). In both species, GA_3 did not show an inductive effect on callogenesis.

3.1. Initiation and pigment content of calluses in different explants

All explants showed the highest rate of callus initiation 8 days after initial culturing to more than 85% (except for anther at 75%), while stem segments in *Rosa gallica* L. initiated calluses (>90%) after 4 days (Figures 2–4). Some explants were browned and did not initiate any calluses within 2–3 weeks, but later callus initiation was surprisingly observed in those anther explants with extensive browning (Figures 2 and 3).

Two-month-old calluses produced from different explants in *Rosa gallica* L. were reddish (Figure 5), but those of *R. hybrida* were greenish (Figure 5).

The content of anthocyanin and chlorophyll increased with increasing of culture time or callus age in both species significantly (Figures 6 and 7). In *Rosa gallica* L., the highest content of these pigments was produced in all calluses derived from vegetative explants compared to flower calluses at day 60 after explanting (Figures 6 and 7). The highest value of chlorophyll and anthocyanin in *R. hybrida* was observed in the 60-day-old calluses obtained from leaf and stem explants compared with the others (Figures 6 and 7). The comparison of anthocyanin and chlorophyll in calluses obtained from the 2 species showed a considerable increase of anthocyanin in calluses derived from explants of *R. gallica*, especially vegetative ones, compared to the

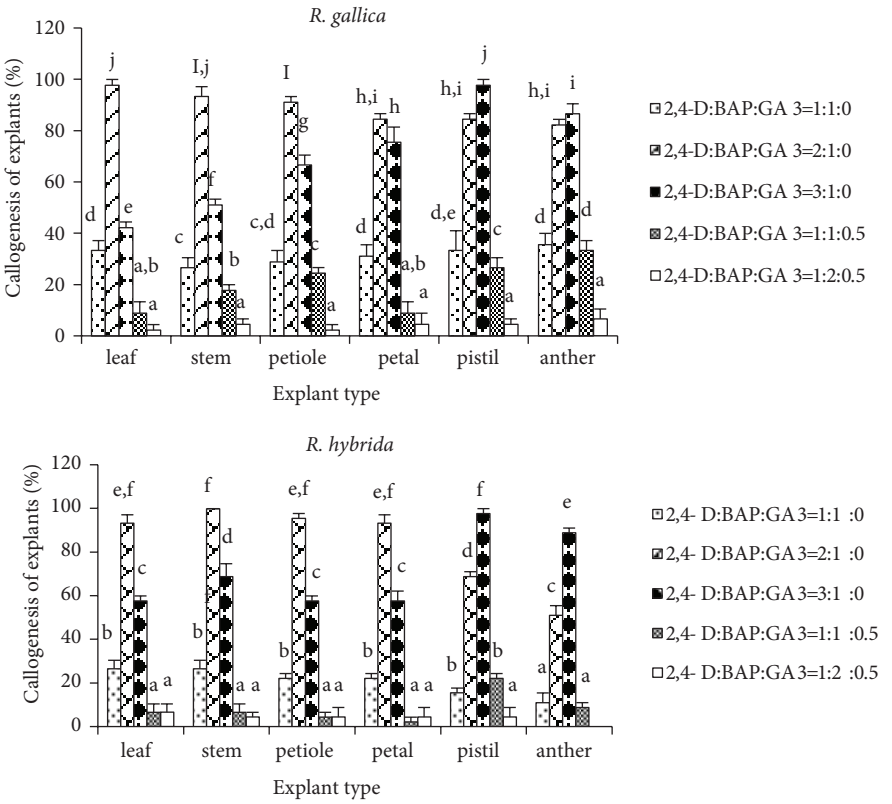

Figure 1. Effect of various growth regulator combinations on callus formation of different explants in *Rosa gallica* and *R. hybrida*. The highest callogenesis (%) was achieved in leaf, stem, petiole, and petal with 2,4-D:BAP:GA_3 = 2:1:0 and in pistil and anther with 2,4-D:BAP:GA_3 = 3:1:0. The level of significance is 5% and data represent means with standard error bars. Letters show significant differences.

Figure 2. Callus initiation of different explants after 4 (A–F) and 8 (G–L) days in *Rosa gallica*. A–F and G–L: stem, leaf, petiole, petal, pistil, and stamen, respectively. The highest rate of callus initiation was seen 8 days after initial culture in all explants, except stem segments, for which it was seen after 4 days. Magnification: A, C, G, and I: 1×, B, D–F, H, and J–L: 2×. Scale bar = 1.0 cm.

same explants in *R. hybrida* (Figures 5 and 8). In contrast, chlorophyll concentration was higher in calluses derived from explants of *R. hybrida* in comparison to *R. gallica* (Figures 5 and 8).

4. Discussion

Vegetative explants showed the highest callogenesis on MS medium containing 2 mg L^{-1} of 2,4-D and 1 mg L^{-1} BAP in both species. The optimal ratio of these growth regulators for pistil and anther was 3:1 mg L^{-1}. Thus, callogenesis is largely based on media formulation and higher concentrations of auxin than cytokinin. The studies of Sakurai et al. (1997) in rose showed that callus was initiated from leaves on an agar-based MS medium supplemented with 5 mg L^{-1} NAA and 0.5 mg L^{-1} BA. In addition, callus development from rose embryos revealed that occurrence was complete only when both BA and NAA were present (Sakurai et al., 1997). Dixon and Gonzales (1996) reported that 2,4-D is a suitable auxin and BAP is used more than other cytokinins for callus induction. Ram et al. (2011) reported that growth and morphogenesis of plant tissue under in vitro conditions are largely governed by culture media composition. Their studies on callusing of leaf and petal explants in *Rosa hybrida* showed that the earliest callus induction (9.67 days) was recorded in leaf explants at a concentration of 4.0 mg L^{-1} 2,4-D, whereas petal explants took longer (11.56 days) to induce callus. Of 2 explants selected, leaf disks were found to be most suitable for callus initiation (Ram et al., 2011). However, in the present study, 2,4-D in combinations with BAP was used and in explants of both species, earliest callus induction was observed at 4 days of culture (except anther in *R. hybrida*), although callusing frequency of vegetative explants was higher than in floral ones. The highest values of callus initiation were

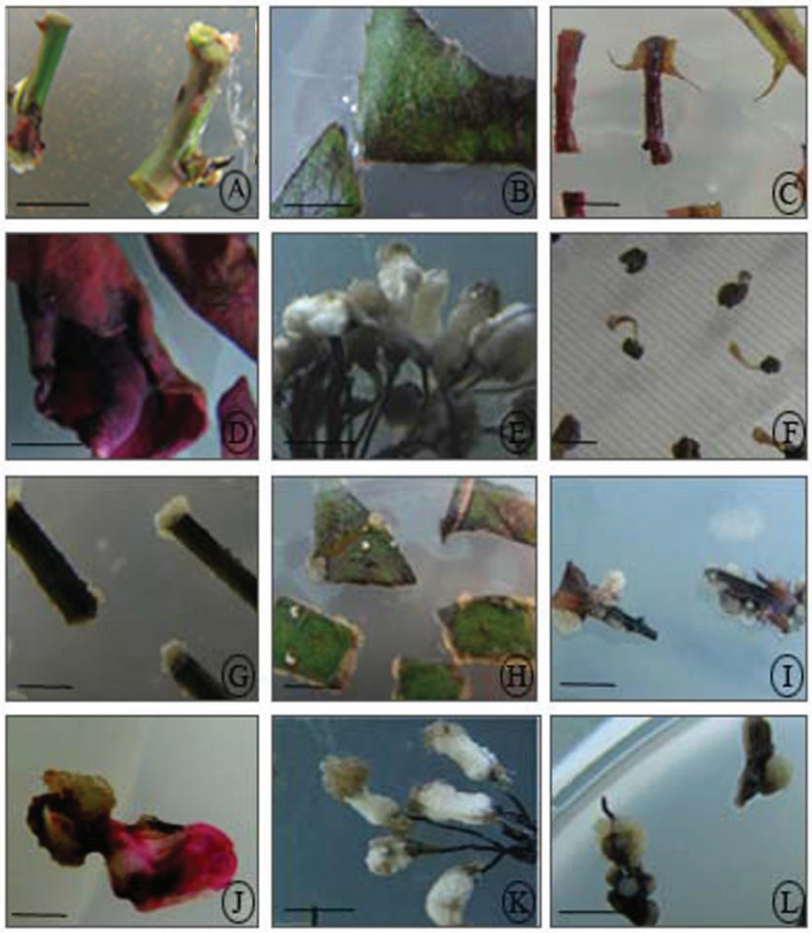

Figure 3. Callus initiation of different explants after 4 (A–F) and 8 (G–L) days in *Rosa hybrida*. A–F and G–L: Stem, leaf, petiole, petal, pistil, and stamen, respectively. The highest value of callus initiation was seen at 8 days of culture in all explants. Magnification: A, B, D, E, K, and L: 4×; F: 2×; and C, G–I, and J: 3×. Scale bar = 1.0 cm.

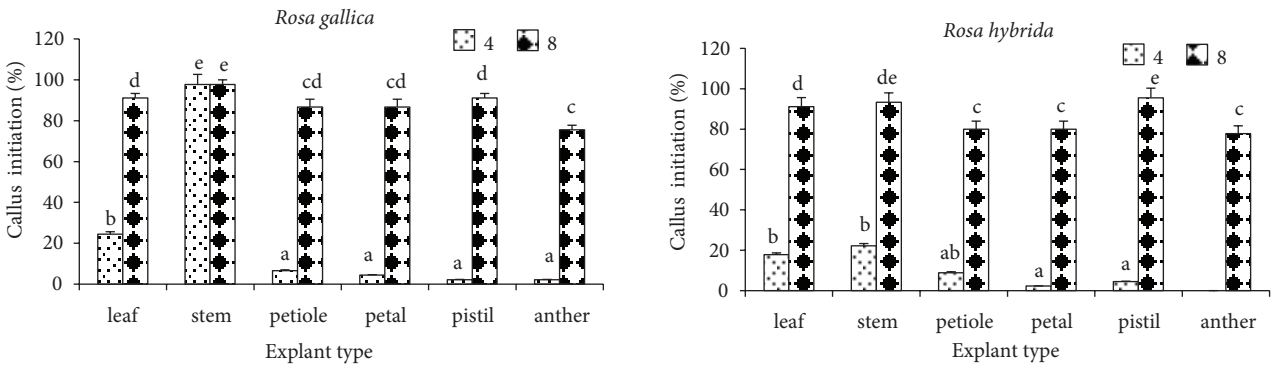

Figure 4. Callus initiation of different explants after 4 and 8 days in *Rosa gallica* and *R. hybrida*. The highest callus initiation (%) was seen in all explants after 8 days for *R. hybrida* but in stem explants was seen after 4 days in *R. gallica*. The level of significance is 5% and data represent means with standard error bars. Letters show significant differences.

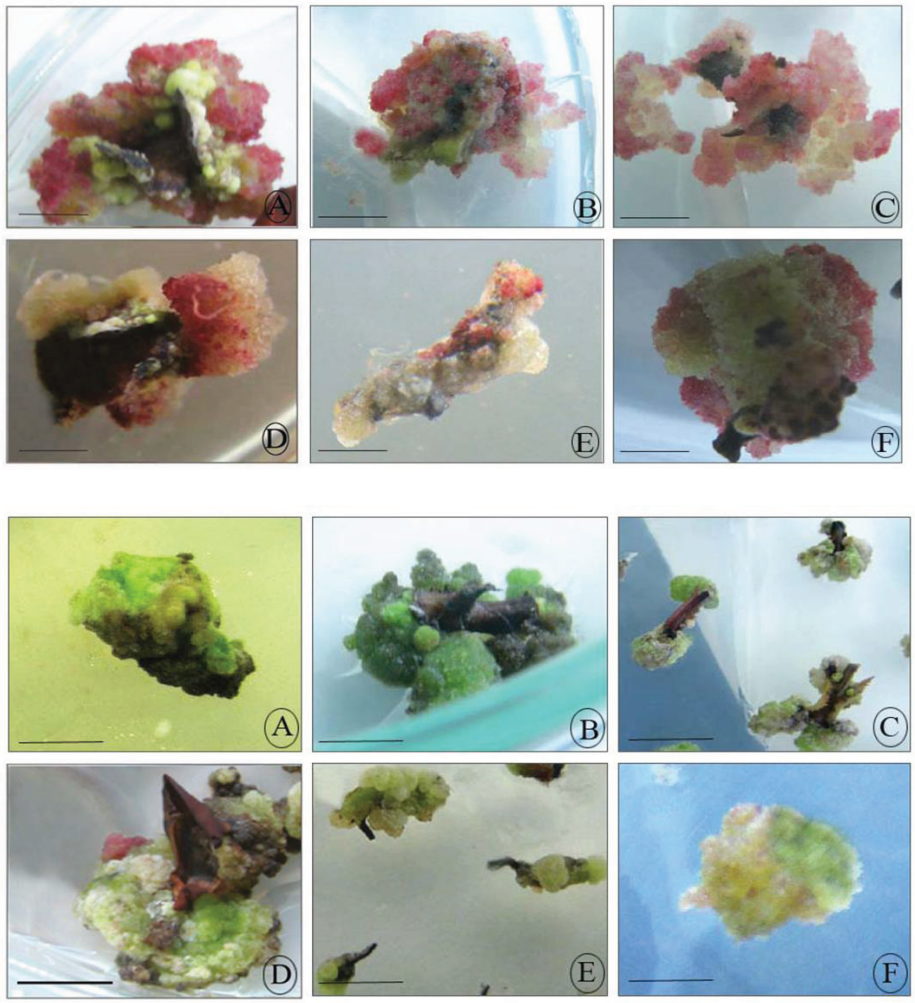

Figure 5. Two-month-old calluses in different explants of *Rosa gallica* and *Rosa hybrida*. A–C: vegetative explants (leaf, stem, and petiole, respectively). D–F: floral explants (petal, pistil, and anther, respectively). The highest content of anthocyanin and chlorophyll pigments was seen in all 60-day-old calluses from vegetative explants in *R. gallica* and 60-day-old calluses from leaf and stem explants in *R. hybrida*. Magnification: A–F: 3×. Scale bar = 1.0 cm.

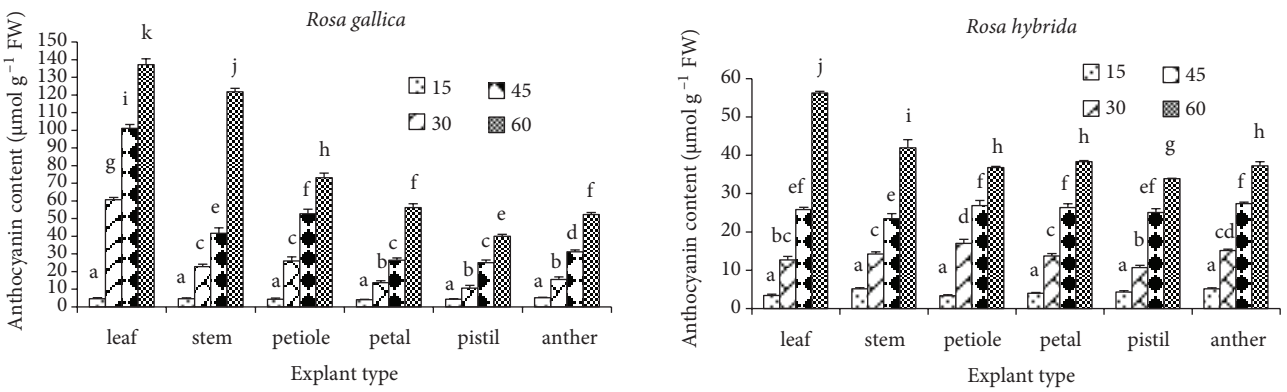

Figure 6. Anthocyanin content of calluses obtained from different explants after 15, 30, 45, and 60 days of culture in *Rosa gallica* and *R. hybrida*. The highest anthocyanin content was seen in all explants after 60 days. The level of significance is 5% and data represent means with standard error bars. Letters show significant differences.

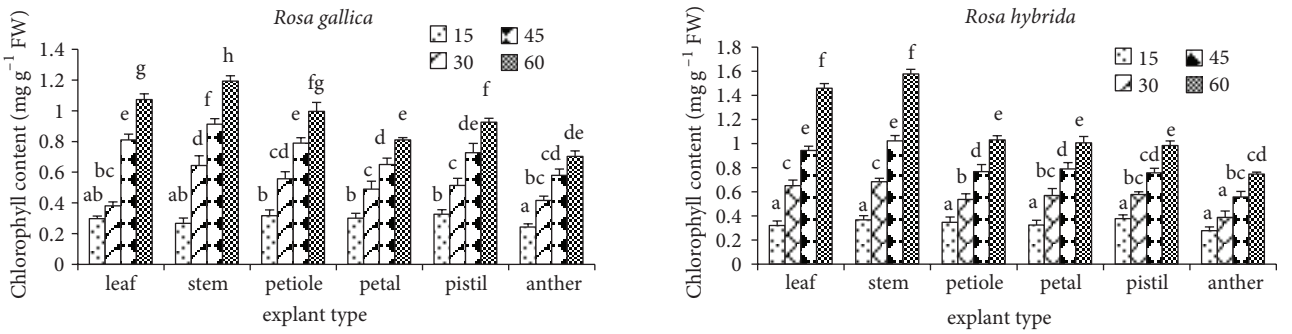

Figure 7. Chlorophyll content of calluses obtained from different explants after 15, 30, 45, and 60 days of culture in *Rosa gallica* and *R. hybrida*. The highest chlorophyll content was seen in all explants after 60 days. The level of significance is 5% and data represent means with standard error bars. Letters show significant differences.

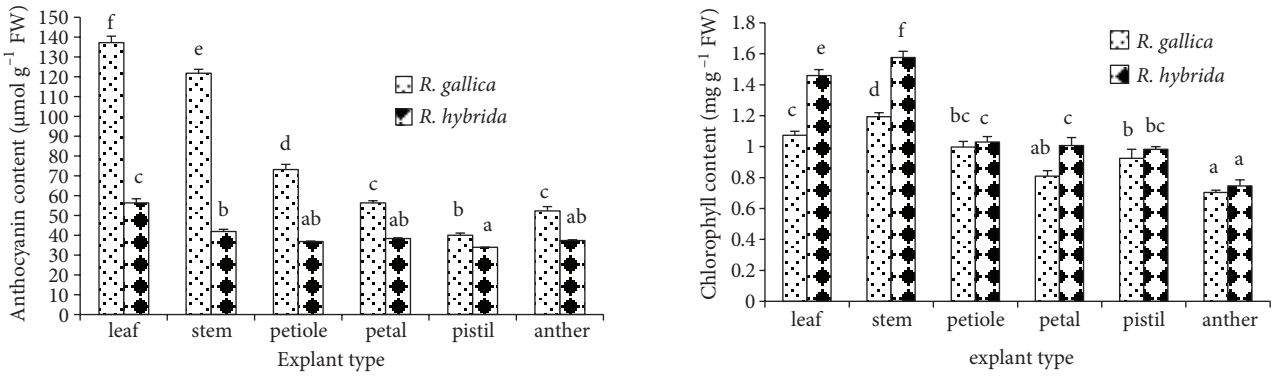

Figure 8. The comparison of anthocyanin and chlorophyll content of calluses obtained from different explants in *Rosa gallica* and *R. hybrida* in 60-day-old calluses. Higher anthocyanin content was seen in all explants of *R. gallica* and higher chlorophyll content in all explants of *R. hybrida*. The level of significance is 5% and data represent means with standard error bars. Letters show significant differences.

recorded during 8 days of culture in all explants. There are many reports on callus formation from vegetative explants as well as the comparison of formation and growth rate of calluses between different explants (Jacob et al., 1969; Lloyd et al., 1988; Burger et al., 1990; Ishioka and Tanimoto, 1990; Rosu et al., 1995; Luciani et al., 2006; Pati et al., 2006). Our literature review revealed that little has been known on callusing of flower explants. The studies of Ram et al. (2011) showed that the highest frequency of callusing (91.11%) was recorded when leaf explants were cultured on MS medium fortified with 4.0 mg L^{-1} 2,4-D, while at the same 2,4-D concentration, the frequency of callusing in petal explants was significantly lower (77.24%). Similarly, the results of this study revealed that callogenesis frequency in vegetative explants was higher compared to floral ones. Callusing ability in anthers was lower than in the other ones. However, it is possible that due to more differentiation in flower explants they initiated calluses with delay and required a higher concentration of 2,4-D for callusing. The differences between explants would indicate that organ differentiation type, totipotency, different levels of endogenous auxins, or other cytoplasmic factors could be involved in these differential abilities.

Anthocyanin and chlorophyll accumulation were increased as time passed from 15 to 60 days. The studies of Blando et al. (2005) in *Prunus cerasus* L. showed that pigment production is absent during the first days of growth in light, while afterwards the pigment accumulation was stimulated by elicitors as light and nutritional factors reached a maximum at nearly the 20th day of culture (Cormier and Do, 1993; Konczak-Islam et al., 2000). Mathur et al. (2010) reported that since anthocyanin accumulation is a cytodifferentiation process, it has been shown to be strongly influenced by culture environment and medium variables. In the present study, the effect of age and species has been surveyed on anthocyanin production. In both species, the highest anthocyanin and chlorophyll yield were obtained in vegetative calluses, especially in the leaf and stem, compared with flower calluses. The comparison of pigment yield in the 2 species showed that in *R. gallica*,

anthocyanin content in vegetative explants was more than 2 times in vegetative explants than that of the same explants in *R. hybrida*. Additionally, in this species an increase of more than 1.5 times the anthocyanin content was observed in petal and anther explants in comparison to those values in *R. hybrida*. In contrast, chlorophyll concentration was somewhat higher in calluses derived from explants of *R. hybrida*, although this increment in petiole, anther, and pistil was not significant. Except for the studies of Ram et al. (2011), there is no published report about the effect of different explant types on pigment content in these species. These researchers, by obtaining only calluses from leaf explants used for anthocyanin induction, considered the early response and higher biomass accumulation. They also did not observe anthocyanin induction in cultures that were grown on MS medium with full-strength NH^{4+} nitrogen (the control). Their studies showed that the highest anthocyanin production was recorded in cultures maintained on MS medium devoid of NH_4^+. On the contrary, the present study showed that anthocyanin production was recorded in cultures grown on MS medium with full-strength NH_4^+ in both species. Furthermore, all explants of the vegetative and floral types produced anthocyanin. Ball et al. (1972) reported that the anthocyanins of the callus cultures in *Dimorphotheca sinuata* D.C. were of the same identity as those in the whole plant, demonstrating that these compounds are produced in the same manner under autophytic and heterophytic conditions. Their results are in conflict with our studies showing a higher concentration of anthocyanin in vegetative explants. There is a possibility of having a high quantity of anthocyanin in vegetative organs in this genus and so further investigations are needed to analyze pigment content in vegetative and floral organs of the whole plant. When an explant is inoculated in vitro, diverse responses are expected and factors determining chemical and physiological characteristics of the tissues donor plants seem to be crucial (Borgatto et al., 2002). According to these researchers as well as the opinion of Ball et al. (1972), in consistence with our results, the higher quantity of chlorophyll in vegetative explants is confirmed because vegetative segments have considerable chlorophyll in the whole plant.

In this study the culture medium for callus induction was optimized. Anthocyanin and chlorophyll contents in calluses produced from vegetative and generative explants of *Rosa hybrida* and *Rosa gallica* were determined. It was found that both species had the greatest callogenesis on MS medium containing a 2–3:1 mg L^{-1} ratio of 2, 4-D to BAP. The highest percentage of callus initiation was obtained after 8 days in all explants of *R. hybrida* and *R. gallica* (except stem explants in *R. gallica*, which initiated the highest callusing after 4 days). The vegetative calluses (respectively leaf, stem, and petiole) showed a higher growth rate in these 2 species. They also showed the highest callus volume after 2 months of plantation. Both anthocyanin and chlorophyll concentration increased with age increment in the 2 species, and the highest content of these pigments was recorded in vegetative calluses, demonstrating that anthocyanin is not produced in the same manner among source plants, in which floral explants, especially petals, have more anthocyanin. In all explants, anthocyanin content in calluses of *R. gallica* was much higher, while chlorophyll concentration was somewhat higher in calluses of *R. hybrida*, and hence calluses in the 2 species were seen as red and green, respectively.

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