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Effect of Sucrose and Abscisic Acid on Anthocyanin Biosynthesis in Rose (*Rosa* × *hybrida*) Callus Cultures

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Abstract

Rose (*Rosa* × *hybrida*) is a highly appealing floral species characterized by a diverse spectrum of colors primarily due to anthocyanin pigments. Anthocyanins are the nutraceutical pigments that are present in roses, having various health-promoting and pharmaceutical properties due to their high antioxidant activities. Contemporary plant tissue culture methods can serve as an alternative for in vitro production of these natural pigments to mitigate seasonal fluctuations. In the present study, efforts were directed toward enhancing anthocyanin pigment production in callus cultures of *Rosa* × *hybrida* cv. Ashwini through the application of different concentrations of sucrose, abscisic acid (ABA), and their combinations. When rose petals were cultured on solid MS medium supplemented with 6.0 mg/l 2,4-dichlorophenoxyacetic acid in complete darkness, early and prolific callus induction was observed. In rose petal calli, the initial pigment induction, enhancement, and increased anthocyanin accumulation were noted when the *Euphorbia millii* (EM) medium contained 80g/l sucrose. Anthocyanin accumulation was augmented in petal-derived rose calli in response to ABA at specific concentrations (30 μM). Increased ABA concentration in the EM medium diminished pigment accumulation. A statistically significant positive correlation was observed between the response coefficient and anthocyanin content (r = 0.960).

Keywords: ABA, Anthocyanins, Nutraceuticals, Rose, Sucrose.

INTRODUCTION

An increasing number of people around the world are looking to phytochemicals as potential new nutraceutical sources. Fruits and vegetables are the only food groups that humans get nutraceutical compounds from (Cavaiuolo et al., 2013). Nevertheless, a number of bioactive compounds found

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in flowers are quickly gaining recognition as potential dietary supplements. They are of interest to human nutritionists because of the strong antioxidant activity suggested by their rich pigmentation. The aglycone anthocyanidin and sugar moieties make up the pigments known as anthocyanins, which dissolve in water (Ockermann et al., 2021). The vibrant hues seen in a wide variety of fruits, flowers, and vegetables are largely attributable to this important class of flavonoids. According to Liu et al. (2018), anthocyanins serve multiple purposes, including reproduction (by luring pollinators and seed dispersers) and defense against abiotic and biotic stresses. Earlier studies have shown that anthocyanin compounds have a high free radical scavenging capacity (Lee et al., 2011; Ge and Ma, 2013; Kumari et al., 2017) and play a vital role in the prevention of cardiovascular disease, obesity, cancer, diabetes, and other diseases (Mattioli et al., 2020; Kumari et al., 2021). Today, interest in anthocyanin pigments has exaggerated because they can be used not only as food and beverage additives to obtain attractive natural red coloration (Roy and Rhim, 2021) but also as nutraceuticals (Hegde et al., 2022; Kumari and Adarsh, 2016; Khoo et al., 2017). Pigment recovery from fresh materials involves constrictions such as variability and seasonal availability of raw materials, fresh material losses, and pigment degradation caused by the extraction process and storage. In our previous study, Kumari et al. (2017) quantified the total anthocyanin content in fifty rose varieties during two different seasons December 2014 and March 2015 and observed the highest anthocyanin accumulation in December 2014 in rose variety Ashwini (578.10 mg/100g on fresh weight basis), which coincided with lower temperature conditions. These findings indicate that anthocyanin content in rose petals is strongly influenced by environmental factors, particularly temperature and light, as well as by the activity of osmotic adjusters during periods of drought and low temperature. Hence, the potential of exploiting anthocyanin pigments for different purposes encouraged the use of plant cells or callus cultures to produce several of these essential secondary metabolites, irrespective of all these hurdles. In vitro culture systems provide a controlled and sustainable alternative for enhanced and consistent production of anthocyanins, independent of climatic or developmental constraints. Moreover, such systems enable optimization of culture conditions and elicitor applications to maximize yield and purity for industrial use. Production of anthocyanin pigments in vitro is well documented in several crops such as *Oxalis reclinata* (Makunga et al., 1997), *Daucus carota* (Sudha and Ravishankar, 2003), *Ipomoea batatas* (Nishiyama and Yamakawa 2004), *Rosa × hybrida* (Hennayake et al., 2006; Ram et al., 2011 and 2013), *Torenia fourunieri* (Nagira et al., 2006), *Cleome rosea* (Simoes et al., 2009), *Hibiscus* *sabdariffa* (Dios-López et al., 2011) and *Bridelia retusa* (Aswathy et al., 2018). However, most of these studies have primarily focused on leaf-derived callus cultures, while investigations on petal-derived callus, particularly in ornamental crops such as rose, remain limited.

Sucrose and abscisic acid (ABA) are key regulators of anthocyanin biosynthesis and play crucial roles in modulating pigment accumulation in plant tissues. Sucrose serves not only as a carbon source but also as an important signalling molecule that regulates the expression of genes involved in the flavonoid biosynthetic pathway. Elevated sucrose levels have been shown to induce the expression of structural genes such as chalcone synthase (CHS), dihydroflavonol reductase (DFR), and anthocyanidin synthase (ANS), thereby enhancing anthocyanin accumulation (Hiratsuka et al.2001; Ferrara et al., 2015). The accumulation of anthocyanins in grape has been shown to be stimulated by the exogenous application of sugars in cell suspension and tissue culture systems (Hiratsuka et al., 2001), possibly as a consequence of osmotic stress (Do and Cormier, 1990).

Abscisic acid (ABA), a stress-responsive phytohormone, is also known to promote anthocyanin biosynthesis by activating transcription factors, particularly those belonging to the MYB family, which regulate key biosynthetic genes (Sun et al., 2017). Furthermore, sucrose and ABA often interact synergistically, suggesting a coordinated regulatory mechanism that integrates metabolic and stress signals to enhance pigment production under in vitro conditions (Bennett et al., 2023). However, to the best of our knowledge, comprehensive studies focusing on the induction and accumulation of anthocyanin pigments in petal-derived callus cultures of rose using these regulatory factors are scarce. Therefore, the present study was undertaken to investigate the induction and accumulation of anthocyanin pigments in petal-derived callus cultures of *Rosa* × *hybrida* cv. Ashwini in response to varying concentrations of sucrose and abscisic acid (ABA), with the objective of developing an efficient in vitro system for enhanced and consistent pigment production.

MATERIALS AND METHODS

Explant and callus induction

Petal explants of *Rosa* × *hybrida* cv. Ashwini were cultured on Murashige and Skoog (MS) basal medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 4.0, 5.0, and 6.0 mg L^{-1} for callus induction. Cultures were maintained at 24 ± 1 °C under a 16/8 h light/dark photoperiod with a light intensity of 105.7 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes.

Cultures were monitored daily to record days to callus initiation, and callusing frequency was expressed as the induction coefficient [% = (the number of explants that form callus / total explants cultured) × 100]. Visual assessment included the site of callus initiation, callus texture (compact, semi-friable, or friable), and callus colour. Callus growth was evaluated after 42 days by measuring fresh weight of callus (FCW) and dry weight of callus (DCW); dry weight was determined after oven drying at 45 °C for 24 h followed by 55 °C for 24 h. Biomass accumulation was estimated using the FCW:DCW ratio. Callus growth performance was categorized into four classes (I–IV) following Matkowski (2004), ranging from weak induction with poor growth to vigorous callus induction and growth.

Multiplication and maintenance of callus from petal explants of *Rosa* × *hybrida* var. Ashwini

The treatment combination showing the highest callus induction (MS medium supplemented with 6.0 mg L^{-1} 2,4-D) was subsequently selected for callus multiplication and long-term maintenance. To support sustained callus growth, the medium was modified by increasing the concentration of MS vitamins to twofold of the standard MS formulation. After 27 days of incubation, the induced calli were transferred from the induction medium to the modified maintenance medium and incubated under complete darkness. Within 7-9 days, the calli were subdivided and exhibited rapid proliferation. The disintegrated mother petal explant was carefully removed to prevent browning due to phenolic exudation. Although removal of the explant initially reduced callus growth, the increased vitamin concentration in the medium effectively restored and supported optimal proliferation.

Sucrose and ABA treatment for accumulation of anthocyanins

Euphorbia millii (EM) medium (Yamamoto et al., 1989) supplemented with sucrose and abscisic acid (ABA) was used for anthocyanin induction. The basal EM medium contains 50 g L^{-1} sucrose (Yamamoto et al., 1998), which served as the reference concentration. Additional treatments were prepared by adjusting sucrose levels to 50, 60, 70, and 80 g L^{-1}. Abscisic acid was dissolved in a small volume of methanol, sterilized by membrane filtration, and added to the autoclaved medium to obtain final concentrations of 20, 30, 40, and 50 µM. Abscissic acid was dissolved in small amount of methanol, sterilized by micro-filtration added into the autoclaved EM medium at 20, 30, 40 and 50 µM concentrations. Combination of sucrose and abscissic acid i.e. 50 g/l + 50 µM, 60 g/l + 40 µM, 70 g/l + 30 µM and 80 g/l + 20 µM were also added to EM medium for initiation of anthocyanin pigments. Before adding agar-agar (5.5 g/l), pH of the medium was adjusted to 5.8 ± 0.1. Prepared media were poured in conical flasks and then autoclaved at 121°C for 16 min at 15 psi. Cultures were incubated in a culture room at 24 ± 1ºC under 16/8 h (105.7 µmol. photons m^{-2} s^{-1} light/dark) photoperiod regime using cool-white fluorescent tubes.

Response coefficient and callus growth index

The anthocyanin biosynthesis in callus culture were estimated for various parameters such as response coefficient (total number of cultures showing pigmentation/ total number of cultured cultures) x 100, number of days taken for pigment initiation and intensification which was visually observed. Days required for pigment initiation denotes the number of days taken for callus tissues to exhibit visible red coloration due to anthocyanin accumulation under the respective culture treatments. Pigment intensification was assessed visually based on progressive color changes from light pink to deep red, increased color saturation, and the spread of pigmentation across the callus tissue. Growth index of pigmented callus was determined based on fresh weight accumulation after 15 days of culture and was used as an indicator of biomass proliferation under different treatments.

Determination of total monomeric anthocyanin content

The total monomeric anthocyanin content was measured by the pH differential method (Wrolstad et al., 2005). A known weight of fresh pigmented callus (500 mg) was extracted with acidified methanol (methanol + 1% HCl) in ultra sonicator water bath for 30 minutes. The extracts were centrifuged at 10,000 rpm for 10 min by using refrigerated centrifuge. Final volume was prepared by adding the acidified methanol (methanol + 1% HCl) to the supernatant. Then final samples were prepared by taking the 5 ml volume of sample and dilutes separately in potassium chloride buffer 0.025 M (pH 1.0) and sodium acetate buffer 0.4 M (pH 4.5) (5ml each). Absorption was measured at 520 and 700 nm after incubation of 15 min at room temperature. A Jasco V 530 UV-Vis spectrophotometer was used for measurements. Absorbance was calculated as $A = [(A510 - A700) \text{ at pH } 1.0] - [(A510 - A700) \text{ at pH } 4.5]$ with a molar extinction coefficient of 26,900 for anthocyanin. The total anthocyanin content was calculated as cyanidin-3-glucoside equivalents as:

Anthocyanin content (mg/ 100 g FW) = $(A \times MW \times DF \times V \times 10^2) / (\varepsilon \times L \times m)$

Where, A is absorbance, MW is the molecular weight of cyanidin-3-glucoside (449.2 Da), DF is the dilution factor, V is the final volume (mL), 10^2 is the factor for conversion from g to mg, ε is the cyanindin-3-glucoside molar absorbance (26,900), L is the cell path length (1 cm), and m is petal weight in g.

Microscopic study of pigmented callus

Microscopic images of the pigmented callus were visualized with a Carl Zeiss Discovery.v8 Stereo microscope (Carl Zeiss MicroImaging GmbH, Berlin, Germany) and images were captured with a Carl Zeiss Axiovision digital camera (software version: Axiovision 4.8.2).

Statistical analysis

The experiment was laid out in completely randomized design (CRD) and data presented in the results are the means ± standard error obtained from at least three independent determinates. All the data were subjected to Turkey's honestly significant difference (HSD) test for the comparison of means and significance for the test was assumed if $P \leq 0.05$. The analyses were carried out through the statistical software SPSS 20.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Effect of 2,4-D Levels on Callus Induction, Multiplication and Biomass Accumulation in *Rosa* × *hybrida* var. Ashwini

The results presented in Table 1 clearly indicate that increasing concentrations of 2,4-D significantly enhanced callus induction and reduced time (days) to callus induction in rose petal explants. The highest induction coefficient (94.67 ± 1.11%) was recorded on MS medium supplemented with 6.0 mg/l 2,4-D, which also promoted the earliest callus initiation and superior callus quality. Thus, 6.0 mg/l 2,4-D was identified as the optimal concentration for efficient callus induction in explants of *Rosa* × *hybrida* cv. Ashwini petal. These findings are in close agreement with Anand et al. (2017), who reported that callus induction on MS medium using leaf explants of bougainvillea cv. Bhabha supplemented with 6.0 mg L^{-1} 2,4-D resulted in the maximum induction coefficient (98.75%) and minimum time required for callus initiation (8.50 days). Similar promotive effects of 2,4-D on callus induction and proliferation have been documented across a range of species and explant types. For instance, El-Nabarawy et al. (2015) identified 2,4-D as the most effective auxin for induction and maintenance of callus cultures in ginger derived from shoot tip explants.

Visual assessment revealed the formation of compact, creamish-white, and vigorously proliferating callus, predominantly originating from the cut and punctured regions of the petal explants (**Fig. 1a**). Progressive callus induction was observed on MS medium supplemented with increasing concentrations of 2,4-D, as evidenced by callus development on 4.0 mg/l (**Fig. 1b**), 5.0 mg/l (**Fig. 1c**), and 6.0 mg/l 2,4-D (**Fig. 1d**), with maximum proliferation recorded at 6.0 mg/l 2,4-D. Higher callusing was observed at both outer and inner wounded sites of the petal explants. Enhanced morphogenic responses in wounded explants have also been reported in other plant species (D'Onofrio and Morini, 2003; Pacheco et al., 2008), likely reflecting variations in endogenous hormonal balance and cellular differentiation among tissue regions (Koroch et al., 2002). The localized initiation of cell proliferation at injury sites may be attributed to the accumulation of auxins at the wound surface, which, in combination with exogenously supplied plant growth regulators, stimulates rapid cell division. This interpretation is consistent with earlier hypotheses suggesting that auxin accumulation at injury sites plays a key role in triggering callus formation (Ahmad et al., 2010). Petal-derived callus cultured on MS medium supplemented with 6.0 mg/l 2,4-D showed progressive multiplication with a continuous increase in biomass during successive culture periods, as observed in 27-day-old (**Fig. 2a**), 50-day-old (**Fig. 2b**), and 65-day-old callus cultures (**Fig. 2c**). The callus remained healthy and proliferative when maintained under complete darkness at 24 ± 1°C (**Fig. 2d**), indicating that these culture conditions were suitable for sustained growth and maintenance.

As indicated by the results (Fig. 3), petal-derived calli exhibited steady growth across the 2,4-D supplemented treatments, with the maximum fresh (490.86 ± 0.66 mg) and dry (98.89 ± 0.99 mg) callus weights recorded on MS medium supplemented with 6.0 mg/l 2,4-D. In contrast, explants cultured on the control treatment consisting of MS basal medium without 2,4-D failed to produce callus, indicating that endogenous hormone levels in the petal explants were insufficient to induce callogenesis under in vitro conditions. The control treatment thus served as a baseline for evaluating the specific effect of exogenous 2,4-D on callus induction and biomass accumulation. These findings confirm the essential role of 2,4-D in initiating and sustaining callus growth in *Rosa* × *hybrida* cv. Ashwini. The strong stimulatory effect of 2,4-D on biomass accumulation may be attributed to its ability to maintain cells in an actively dividing, dedifferentiated state. Similar findings have been reported by Duangporn and Siripong (2009), who observed a greater influence of 2,4-D on callus growth compared with NAA. The present results are also consistent with earlier reports in rose and bougainvillea, where higher concentrations of 2,4-D promoted increased callus biomass (Ram, 2015; Anand et al., 2017). Furthermore, synergistic interactions between 2,4-D and cytokinins such as BA have been shown to enhance callus fresh and dry weights, emphasizing the importance of auxin–cytokinin balance for optimal callus proliferation (Verma et al., 2012; El-Nabarawy et al., 2015).

To evaluate actual biomass accumulation, the fresh-to-dry callus weight (FCW:DCW) ratio, an indicator of tissue water content, was calculated (Fig. 4). Although callus tissues generally contain a high proportion of water, the lowest FCW:DCW ratio (4.83 ± 0.14) was observed in calli cultured on MS medium with 6.0 mg/l 2,4-D, indicating the formation of compact, non-watery callus with superior dry matter accumulation. Similar trends have been reported earlier (Ram, 2015; Anand et al., 2017). In contrast, explants cultured on growth-regulator-free MS medium failed to produce callus, underscoring the essential role of exogenous auxin in initiating and sustaining callus growth.

Table 1. Effect of 2,4-D levels on callus induction in petals explants of *Rosa* × *hybrida* cv. Ashwini.

Treatment	**Induction coefficient per cent (%)**	**Days to callus induction**	**Callus growth status**
T0 (MS + No hormone)	0.00	0.00	-
T1 (MS+2,4-D 4mg/l)	63.33±0.53^{c} (52.71)	13.33±0.33^{a}	II
T2 (MS+2,4-D 5mg/l)	85.67±0.71^{b} (67.74)	11.33±1.20^{a}	III
T3 (MS+2,4-D 6mg/l)	94.67±1.11^{a} (76.70)	8.67±0.67^{b}	IV
SEm±	0.71	0.76	
CD (P≤0.05)	2.36	2.31	

*Values in parenthesis are arc sin transformed values
I = Weak callus initiation and poor growth.
II = Good induction of callus but poor growth.
III = Good initiation and moderate growth.
IV = Best induction and vigorous growth of callus.

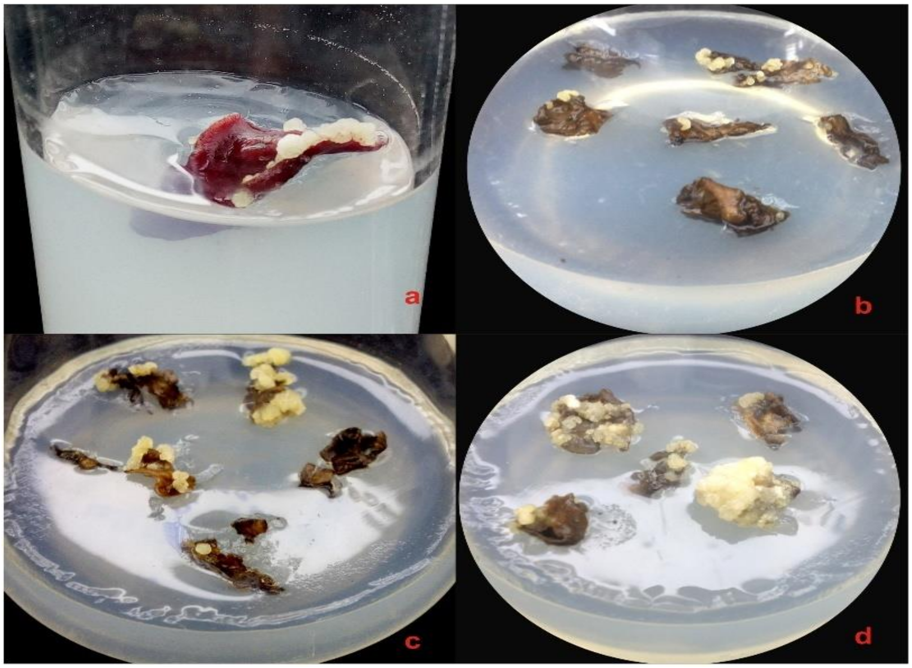

Fig. 1. Callus induction from petal explant of *Rosa* × *hybrida* var. Ashwini: **(a)** Callus initiation from petal explant **(b)** Callus induction on MS medium supplemented with 4 mg/l 2,4-D **(c)** Callus induction on MS medium supplemented with 5 mg/l 2,4-D **(d)** Callus induction on MS medium supplemented with 6 mg/l 2,4-D.

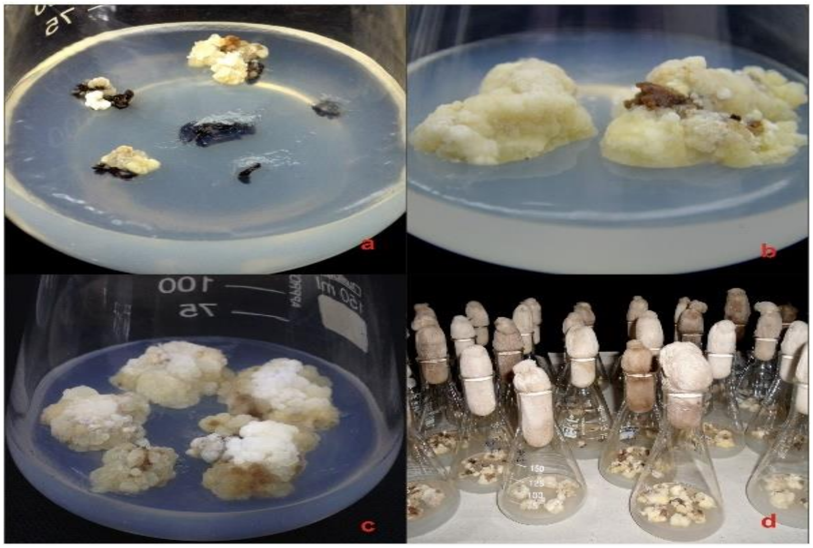

Fig.2. Multiplication of petal derived callus of *Rosa* × *hybrida* in MS medium supplemented with 6 mg/l 2,4-D. **a.** 27 days old callus; **b.** 50 days old callus, **c.** 65 days old callus **d.** callus maintenance in complete darkness at 24±1^{o} C.

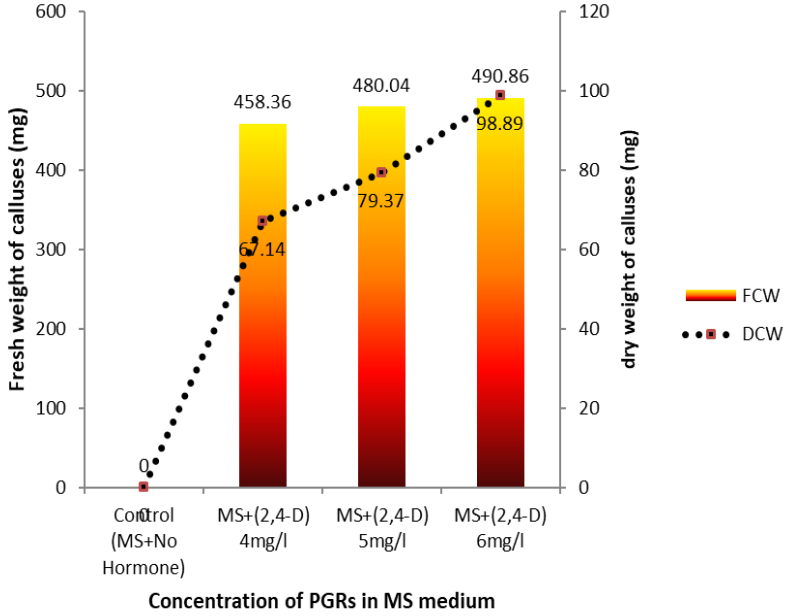

Fig. 3. Effect of 2,4-D levels on callus fresh weight and dry weight of *Rosa* × *hybrida* var. Ashwini.

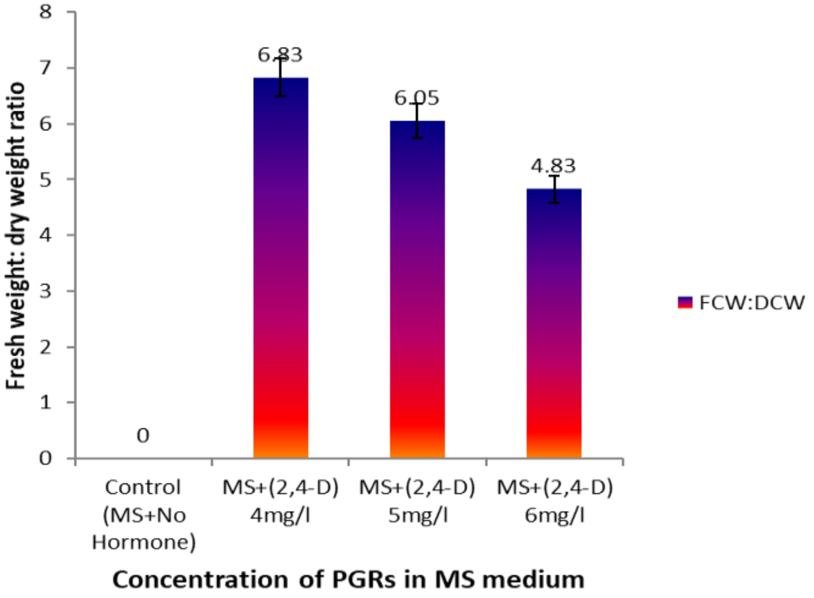

Fig. 4 Effect of 2,4-D levels on fresh weight: dry weight ratio of callus.

Effect of Sucrose and ABA treatment on anthocyanin production

The stimulation of anthocyanin biosynthesis in plant cell cultures has been shown to depend on numerous factors, such as growth regulators, light, temperature, phosphate, nitrate, sugar and initial inoculum density (Sato et al., 1996; Zhang et al., 1997; Takeda et al., 2003). In our investigation we have observed that increasing the sucrose level in solid medium invariably led to higher anthocyanin accumulation in rose callus derived from petal explants (Table 2). Maximum response coefficient percentage was registered with EM medium supplemented with sucrose 80 g/l (T4) (67.03%) followed non-significantly by EM + sucrose 70 g/l (T3) (64.43%) and EM + sucrose 60 g/l (T2) (57.22%). Minimum response coefficient percentage was established in control (T0) (32.78%). The onset of anthocyanin induction in petal-derived callus was initially inferred from gradual color changes from creamish-white to light pink or reddish tones following transfer to induction media. Earliest pigment induction (5.33±0.6 days) was observed when 80 g/l sucrose (T4) was supplemented followed non-significantly by EM + sucrose (70 g/l) + ABA (30 µM) (T11) (7.00±1.15 days) and EM + sucrose (80 g/l) + ABA (20 µM) (T12) 7.33 days). Anthocyanin induction was slow when EM medium supplemented with sucrose 50 g/l (T1) (17.33±1.20 days). Anthocyanin intensification was earliest (8.67±1.20 days) when EM medium supplemented with sucrose 80 g/l (T4) followed non- significantly by EM + sucrose (80 g/l) + ABA (20 µM) (T12) (9.33±0.88 days) and EM + sucrose (70 g/l) + ABA (30 µM) (T11) (9.67±0.67 days). Pigmentation was delayed maximum (19.67±0.67 days) when sucrose was used at 50 g/l (T1). Increasing sucrose concentration and the combined application of sucrose and ABA resulted in higher response coefficient values and enhanced pigment intensification. The augmentative effects of sugars and ABA on anthocyanin accumulation have been reported mainly in the cultured callus of various species (Simões et al., 2009; Chan et al., 2010; Ram et al., 2011). Further, these results showed that anthocyanin biosynthesis in rose callus was associated with the supplementation of sucrose concentration in the EM medium. Results of the present investigation suggest that the addition of sucrose up to an optimum level in the culture medium enhanced anthocyanin biosynthesis in callus cultures of *Rosa* × *hybrida* var. Ashwini. Although sucrose is an indispensable nutrient, it also acts as an osmotic agent when used at high concentrations (Nagira and Ozeki, 2004). Therefore, increased sucrose levels in the medium may have imposed moderate osmotic stress on the cultured tissues, thereby activating physiological responses that stimulated the synthesis of secondary metabolites, particularly anthocyanins.

Similarly, supplementation with abscisic acid (ABA) may have further enhanced pigment accumulation through its role in stress adaptation and regulation of physiological responses in plant tissues. Under such conditions, osmotic stress may also promote the formation of reactive oxygen species such as hydrogen peroxide, which can induce anthocyanin accumulation due to their antioxidant function in reducing oxidative damage. Thus, the combined effects of sucrose and ABA likely created favourable stress-related physiological conditions for enhanced anthocyanin production in rose callus cultures (Olivares et al., 2017; Bennett et al., 2023).

Quantification of anthocyanin pigments

Total anthocyanin content in calli ranged from 0.52 ± 0.06 mg/100 g FW in T0 (EM medium without sucrose supplementation) to 5.34 ± 0.23 mg/100 g FW in T4 (EM medium supplemented with 80 g/l sucrose) (Fig. 5). Quantification of pigment content in calli was highest in treatment T4 (5.34 ± 0.23 mg/100 g FW), corresponding to EM medium supplemented with 80 g/l sucrose, followed non-significantly by treatment T3 containing 70 g/l sucrose (5.15 ± 0.13 mg/100 g fresh weight). Among the treatments, T3, T4, T6, and T12 recorded higher anthocyanin content. Treatment T4 was statistically at par with T3, T3 was at par with T12, and T12 was at par with T6, indicating no significant difference among these treatments and suggesting that both individual treatments (sucrose or ABA alone) and their combined application were effective for anthocyanin induction. These findings suggest that multiple treatment strategies may be utilized for enhanced pigment production in rose callus cultures. Stereomicroscopic observations (40×) revealed comparative visual differences in red pigmentation intensity among petal-derived callus cultures of *Rosa* × *hybrida* var. Ashwini under different treatments, indicating variation in anthocyanin accumulation (Fig. 6a–f). Among all treatments, callus tissues proliferated on 80 g/L sucrose (Fig. 6c) exhibited the most intense red pigmentation, suggesting enhanced anthocyanin biosynthesis compared with the other treatments. The present experiment showed earliest and higher accumulation of anthocyanins in response to optimum sucrose level (80 g/l), which may be attributed to the high osmotic potential of culture and in response to that the cultures biosynthesized the secondary metabolite, i.e. anthocyanins. The present findings are supported by number of researchers. Higher (6-7%) level of sucrose in culture medium resulted more accumulation of anthocyanin in *Vitis vinifera* (Cormier et al., 1990) and *Rosa* × *hybrida* cv. ‘Charleston’ (Hennayake et al., 2006). Sucrose increases the osmotic potential of culture medium which imposes the osmotic stress in the cultures, thereby inducing the anthocyanin biosynthesis (Hennayake et al., 2006; Mathur et al. 2010; Ram et al. 2011). Anthocyanin accumulation was promoted in cell suspension culture *of Melastoma malabathricum* using sucrose as osmoticum (See et al. 2011). Our results are in accordance with the findings of Ram et al. (2011), they found that the medium containing 7% sucrose led to the higher anthocyanin induction in callus cultures of *Rosa* × *hybrida*. They suggested that osmotic-stress imposed by high sucrose levels favoured production of anthocyanins. In anthocyanin biosynthesis, sugars could act as precursors of metabolic processes and as a signalling molecule for promoting gene expression (Solfanelli et al., 2006; Weiss, 2000). Genes coding for dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS) were up-regulated and the accumulation of anthocyanins was strongly increased by sucrose in grape cells (Gollop et al., 2002). Dai et al. (2013) suggested that the sugar-induced enhancement of anthocyanin accumulation results from altered expression of regulatory and structural genes (especially UDP-glucose: anthocyanidin 3-O-glucosyltransferase), together with massive reprogramming in signalling transduction pathways. Lowest anthocyanin content (0.52±0.06 mg/100 g fresh weight) was recorded in control (EM+0). Pigment content increased to 4.67±0.15 mg/100 g FW when EM medium supplemented with EM + ABA (30 µM). However, if ABA concentration increases in the EM medium beyond (30 µM); pigment content decreases (Fig. 5). Abscisic acid was reported to show enhancing effects on the synthesis of anthocyanin in many plant species like tomato hypocotyl (Carvalho et al., 2010), grapevine leaves (Pirie and Mullins, 1976) and torenia shoots (Nagira et al., 2006) and our results are in accordance with that. ABA may enhance anthocyanin accumulation through stress-related signaling as well as through induction of key biosynthetic enzymes and regulatory genes involved in the anthocyanin pathway. This is supported by an increase in the expression of the UFGT (UDP- glucose: flavonoid-3-O-glycosyl tranferase coding for a specific to the anthocyanin pathway) and VvMYBAl genes (coding for a transcriptional regulator controlling anthocyanin biosynthesis), as well as other genes coding upstream located enzymes i.e. PAL-phenylalanine ammonia-lyase, CHI-chalcone isomerase, CHS- chalcone synthase (Gagné et al., 2011; Ma et al., 2024). Furthermore, ABA has been reported to induce PAL activity, a key rate-limiting enzyme of the phenylpropanoid pathway that plays a crucial role in the biosynthesis of anthocyanins and other phenolic compounds, thereby significantly enhancing pigment accumulation (Koyama et al., 2010).

From Fig. 7, it is evident that the maximum fresh weight (FCW) of callus was observed in the control (T0) (396.71 ± 1.25 mg), followed by EM + 50 µM ABA (T8) (324.03 ± 5.42 mg). The minimum fresh weight (273.64 ± 0.68 mg) was recorded in EM + 80 g/L sucrose (T4). These results indicate that increasing sucrose concentration in the culture medium significantly reduced callus growth and biomass accumulation. The maximum biomass accumulation was recorded in the control (EM medium only), whereas the minimum was observed under high sucrose treatment. This reduction in growth at elevated sucrose levels may be attributed to increased osmotic stress in the culture medium, which could limit nutrient uptake and thereby suppress callus proliferation. Similar findings were reported by Sato et al. (1996), who observed decreased cell growth in strawberry suspension cultures under high sucrose concentrations due to increased osmotic potential or medium viscosity.

However, when sucrose (80 g/L) and ABA (20 µM) (T12) were applied in combination, a pronounced synergistic effect on anthocyanin production was observed, indicating that the combined application of optimum sucrose and ABA levels positively influenced pigment accumulation compared with 20 µM ABA alone. These findings are in agreement with Hiratsuka et al. (2001).

Correlation among parameters of anthocyanin induction by sucrose and ABA in petal-derived calli of *Rosa* × *hybrida* var. Ashwini

A statistically significant positive correlation was observed between the response coefficient and anthocyanin content (r = 0.960) (Table 3), indicating that higher responsiveness of callus tissue was associated with greater pigment accumulation. In contrast, a negative correlation was found between the response coefficient percentage and the days required for pigment initiation and intensification, suggesting that calli exhibiting early pigment induction tended to have higher anthocyanin productivity. Similarly, days required for pigment initiation and intensification were negatively correlated with pigment content, reflecting that prolonged induction periods resulted in reduced pigment accumulation. Interestingly, a significant positive correlation was detected among days required for pigment initiation, pigment intensification, and fresh weight of callus, implying that slower pigment development might coincide with enhanced biomass accumulation. This inverse relationship between pigment biosynthesis and growth has been previously reported in various plant tissue cultures, where secondary metabolite production often occurs at the expense of cell proliferation (Namdeo 2007; Ram et al. 2011, 2013). A non-significant negative correlation between anthocyanin content and fresh cell weight (r = –0.891) further supports this trend. Overall, these correlations highlight the metabolic trade-off between growth and anthocyanin biosynthesis in *Rosa* × *hybrida* callus cultures under sucrose and ABA treatments. Similar interactions between carbon source concentration, hormonal regulation, and anthocyanin accumulation have been reported in *Fragaria* × *ananassa* (Xu et al., 2014), and *Petunia hybrida* (Albert et al., 2014), reinforcing the role of sucrose and ABA as key regulators of anthocyanin biosynthesis.

Table 2 Effect of sucrose and ABA on anthocyanin pigment production, days to pigment initiation, and days to pigment intensification in callus cultures of *Rosa* × *hybrida* var. Ashwini.

Treatment	**Response coefficient (%)**	**Pigment initiation (Days)**	**Pigment intensification (Days)**
T0=EM + 0 (control)	29.33±0.55^{h} (32.78)	15.67±0.88^{a}	17.67±0.33^{a}
T1=EM + sucrose (50 g/l)	51.00±2.84^{fg} (45.56)	17.33±1.20^{a}	19.67±0.67^{a}
T2=EM + sucrose (60 g/l)	70.33±3.87^{cd} (57.22)	10.67±0.67^{bcd}	13.67±2.40^{b}
T3=EM + sucrose (70 g/l)	81.33±1.39^{ab} (64.43)	7.33±0.88^{de}	10.67±1.45^{bcd}
T4=EM + sucrose (80 g/l)	83.67±5.07^{a} (67.03)	5.33±0.66^{e}	8.67±1.20^{d}
T5=EM + ABA (20 µM)	53.67±0.83^{fg} (47.08)	8.49±1.81^{cde}	12.33±0.88^{bcd}
T6=EM + ABA (30 µM)	71.66±1.13^{cd} (57.84)	8.17±2.18^{cde}	11.67±1.764^{bcd}
T7=EM + ABA (40 µM)	44.33±1.26^{fg} (41.72)	10.82±0.62^{bcd}	12.33±0.66^{bcd}
T8=EM + ABA (50 µM)	43.33±0.97^{g} (41.15)	10.67±1.20^{bcd}	13.67±0.67^{b}
T9=EM + sucrose (50 g/ l) + ABA (50 µM)	50.33±1.01^{fg} (45.17)	11.33±1.20^{bc}	13.66±1.86^{b}
T10=EM + sucrose (60 g/l) + ABA (40µM)	54.67±1.12^{ef} (47.66)	12.00±1.00^{b}	13.33±1.20^{bc}
T11=EM + sucrose (70 g/l) + ABA (30 µM)	64.33±0.72^{de} (53.31)	7.00±1.15^{e}	9.67±0.67^{cd}
T12=EM + sucrose (80 g/l) + ABA (20 µM)	75.000±0.76^{bc} (59.99)	7.33±1.20^{de}	9.33±0.88^{d}
SEm±	2.12	1.21	1.26
CD (P≤0.05)	6.21	3.53	3.69

*Values in parenthesis are arc sin transformed values.

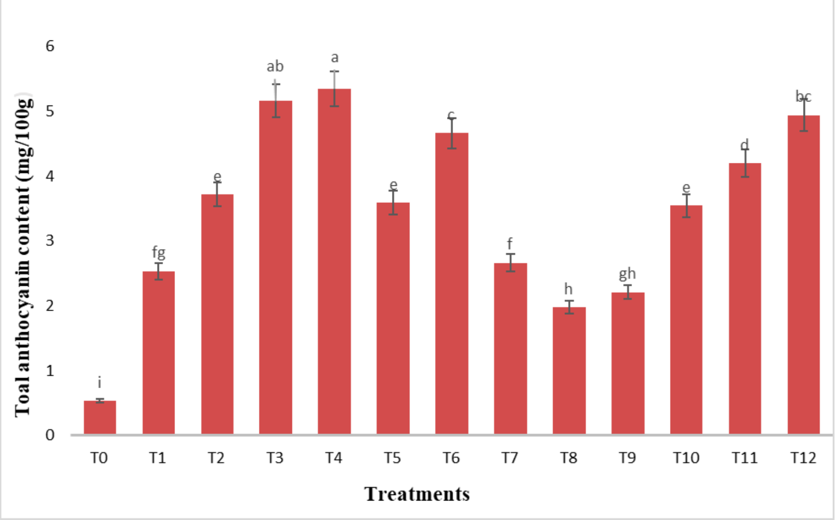

Fig. 5. Effect of sucrose and ABA on anthocyanin content in callus cultures of *Rosa* × *hybrida* var. Ashwini, recorded two weeks after culture initiation on pigment induction (EM) medium.
T0=EM + 0 (control), T1=EM + sucrose (50 g/l), T2=EM + sucrose (60 g/l)
T3=EM + sucrose (70 g/l), T4=EM + sucrose (80 g/l) , T5=EM + ABA (20 µM)
T6=EM + ABA (30 µM) , T7=EM + ABA (40 µM) , T8=EM + ABA (50 µM)
T9=EM + sucrose (50 g/l) + ABA (50 µM) , T10=EM + sucrose (60 g/l) + ABA (40 µM)
T11=EM + sucrose (70 g/l) + ABA (30 µM), T12=EM + sucrose (80 g/l) + ABA (20 µM)
The same letter on the bar graph did not differ significantly at 5% level of significance when compared by Tukey's HSD test.

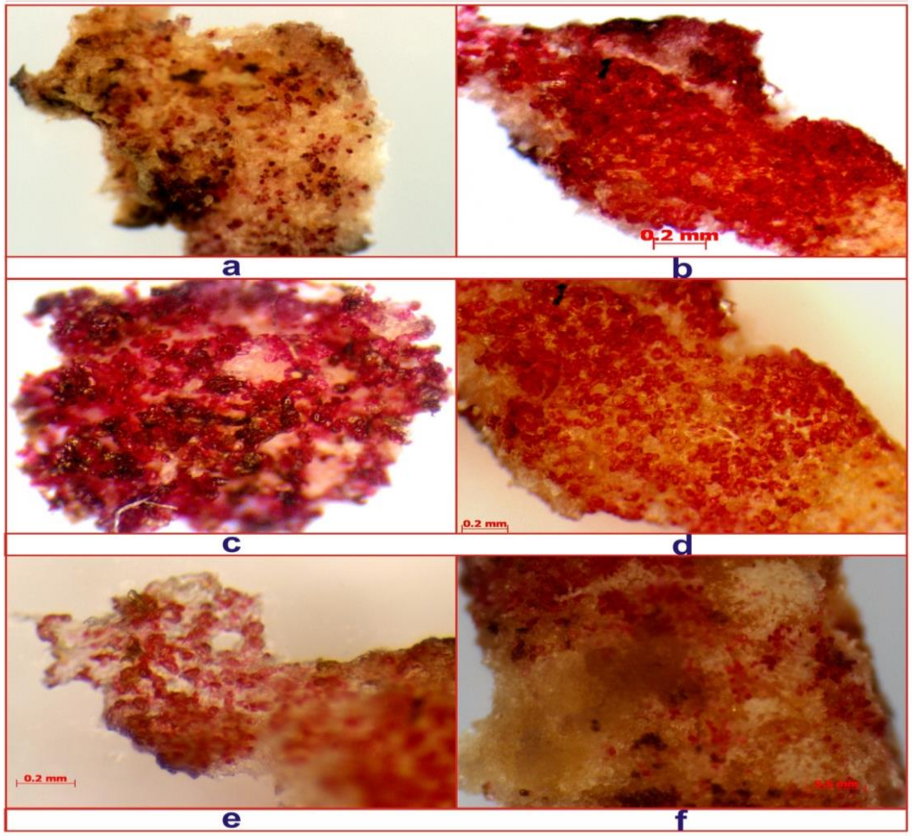

Fig. 6. Stereomicroscopic (40×) view showing comparative visual differences in red pigmentation intensity in petal-derived callus cultures of *Rosa* × *hybrida* var. Ashwini, recorded two weeks after culture initiation on pigment induction (EM) medium supplemented with respective treatments.

a. T0=EM + 0 (control)
b. T3=EM + sucrose (70 g/l)
c. T4=EM + sucrose (80 g/l)
d. T6=EM + ABA (30 µM),
e. T11=EM + sucrose (70 g/l) + ABA (30 µM)
f. T12=EM + sucrose (80 g/l) + ABA (20 µM)

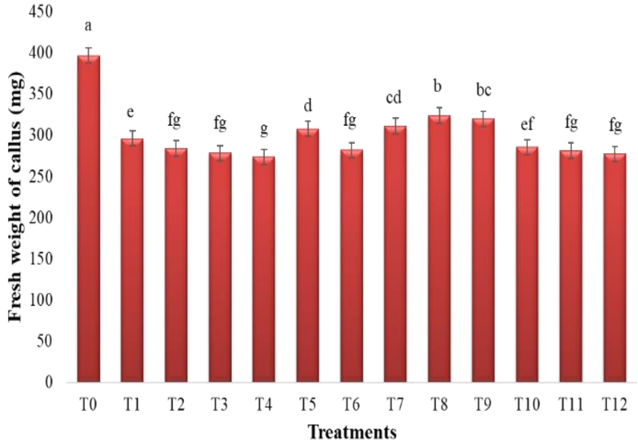

Fig. 7. Effect of sucrose and ABA on fresh weight of callus in *Rosa* × *hybrida* var. Ashwini, recorded two weeks after culture initiation on pigment induction (EM) medium supplemented with respective treatments.

T0=EM + 0 (control), T1=EM + sucrose (50 g/l), T2=EM + sucrose (60 g/l)
T3=EM + sucrose (70 g/l), T4=EM + sucrose (80 g/l) , T5=EM + ABA (20 µM)
T6=EM + ABA (30 µM) , T7=EM + ABA (40 µM) , T8=EM + ABA (50 µM)
T9=EM + sucrose (50 g/l) + ABA (50 µM) , T10=EM + sucrose (60 g/l) + ABA (40 µM)
T11=EM + sucrose (70 g/l) + ABA (30 µM), T12=EM + sucrose (80 g/l) + ABA (20 µM)
The same letter on the bar graph did not differ significantly at 5% level of significance when compared by Tukey's HSD test.

Table 3 Correlation between different parameters recorded for anthocyanin induction by sucrose and ABA in petal derived calli of *Rosa* × *hybrida* var. Ashwini.

Parameters	**RCP**	**NDTP Initi.**	**NDTP Inten.**	**AC**	**FCW**
RCP	1	-0.754**	-0.713**	0.960**	-0.849**
NDTP Initi.		1	0.976**	-0.813**	0.612*
NDTP Inten.			1	-0.790**	0.608*
AC				1	-0.891**
FCW					1

RCI= Response coefficient percentage, NDTP Initi.=Number of days taken for pigment initiation, NDTP Inten.= Number of days taken for pigment intensification, FCW=Fresh cell weight, AC=Anthocyanin content
$^{*, **}$Correlation is significantly different at P≤ 0.05 and 0.01 respectively

Conclusion

The present study demonstrated that anthocyanin induction in petal-derived callus cultures of *Rosa* × *hybrida* cv. Ashwini can be effectively enhanced through manipulation of sucrose and ABA levels in EM medium. Among the treatments tested, EM medium supplemented with 80 g L^{-1} sucrose produced the highest anthocyanin accumulation, although it was statistically at par with 70 g L^{-1} sucrose. ABA alone also promoted anthocyanin biosynthesis, with 30 µM ABA being the most effective among the ABA treatments. The combined application of sucrose and ABA accelerated pigment induction and intensification, indicating a synergistic influence on anthocyanin biosynthesis. Overall, EM medium supplemented with high sucrose concentrations (70-80 g L^{-1}), either alone or in combination with ABA, represents an effective in vitro system for enhanced anthocyanin production in rose callus cultures. While this optimized protocol demonstrates potential for research-scale pigment induction and mechanistic studies, further work using natural or GRAS (Generally Recognized as Safe) elicitors would be necessary to develop a process suitable for food, nutraceutical, or pharmaceutical applications.

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