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Influence of biotic and abiotic elicitors on production of betalain pigments in bougainvillea callus cultures

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ABSTRACT

The present study was carried out to investigate the effects of various biotic and abiotic elicitors on biosynthesis of betalain pigments in bougainvillea callus cultures. Addition of different concentrations of biotic elicitors (methyl jasmonate [MJ] and β-glucan) and abiotic elicitors (calcium chloride, FeEDTA and copper sulphate) into basal Murashige and Skoog's (MS) medium influenced betacyanin and betaxanthin production. Treatment with 0.5 µM MJ was found to be most effective in inducing betalain biosynthesis in the callus. Maximum response coefficient (81.25%), earliest pigment initiation (7 days) and intensification (13.75 days) was observed with 0.5 µM MJ. Beyond this concentration there was a decrease in response coefficient and pigment content. Among the different concentrations of β-glucan, 0.5 mg/l was most effective in increasing the betacyanin and betaxanthin contents (0.35 and 0.22 mg/g FW respectively). Among the abiotic elicitors, calcium chloride at 5 g/l showed maximium response coefficient (78.75%), betacyanin (0.61 mg/g FW) and betaxanthin content (0.42 mg/g FW). FeEDTA at 100 µM and $CuSO_4$ at 20 µM showed good response coefficient with higher betaxanthin and betacyanin content but at higher concentrations both the response coefficient and pigment production decreased. Correlation between response coefficient, betacyanin and betaxanthin content was also calculated.

Key words: Betacyanin, betaxanthin, callus, biotic elicitors, abiotic elicitors, bougainvillea.

INTRODUCTION

Betalains comprise a class of nitrogen- containing plant pigments found in the cell sap of plants representing most families of the Caryophyllales and some higher fungi. This group of pigments has a wide range of colours from red-violet betacyanins to yellow betaxanthins, is water-soluble and non-toxic. Interest in these molecules has grown since their antioxidant and free radical scavenging properties were characterized (Kanner *et al*., 8). Plant cell and tissue cultures are attractive alternative sources of bioactive plant substances, including betalain pigments (Rao and Ravishankar, 12). Elicitation can be an important strategy towards improved *in vitro* production of plant secondary metabolites. Elicitors are compounds of mainly microbial origin or non-biological origin. In cell and organ cultures, biotic and abiotic elicitors have effectively stimulated the production of plant secondary metabolites from almost all chemical classes. However, there is very little information regarding the effects of elicitors on production of betalain pigments in cell cultures. Furthermore, until now there is no report available regarding the effect of elicitors on betalain production in callus cultures of *Bougainvillea* spp. Therefore, the present study was undertaken.

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MATERIALS AND METHODS

Callus used in this investigation was originally derived from leaf explant of bougainvillea cv. Bhabha cultured on the MS basal medium (Murashige and Skoog, 9) supplemented with 6 mg/l 2,4-D and it was continuously maintained on the same medium with double the quantity of vitamins at 24 ± 1°C in complete darkness. Stock callus cultures were maintained under the same physical conditions described above that were sub-cultured at 21-day interval. Stock solutions of different elicitors and methyl jasmonate (MJ) were prepared and sterilized by filtration (0.22 µm; Millipore®, USA) before addition to standard MS medium. Medium pH was adjusted to 5.8 ± 0.1 prior to adding agar-agar (5.5 g l^{-1}, Qualigens Chemicals, Mumbai), autoclaved (121°C) for 15 min. and dispensed into test tubes (25 ml). Cultures were incubated in a culture room at 24 ± 1°C under 16/8 h (105.7 µmol photons $m^{-2}s^{-1}$ light/dark) photoperiod regime using cool-white fluorescent tubes. Basal MS medium was used as control.

The betalain biosynthesis in callus cultures was measured by different parameters such as response coefficient = (total number of cultures showing pigmentation/ total number of cultured cultures)

× 100, number of days taken for pigment initiation and intensification which was visually observed. Extraction and quantification of betalains from bougainvillea callus tissue was carried out based on the method described by Castellanos-Santiago and Yahia (3) with minor modifications. Proliferated callus masses were harvested to measure the betacyanin and betaxanthin contents. Pigmented and non-pigmented callus samples (100 mg) were macerated using double-distilled water. The extracts were centrifuged at 12000 × g for 10 min. in a refrigerated (4°C) centrifuge (Sigma 3K30, Germany). Optical density (OD) of the supernatant of each sample was measured at 483 and 535 nm using a UV-vis double-beam spectrophotometer (Thermo Electron Corp.,USA) against the blank which consisted of double-distilled water

The experiment was laid out in completely randomized design with four replications. Data were analyzed using one-way analysis of variance (ANOVA). Significance for the test was assumed at $P \leq 0.05$. Correlation between response coefficient, betacyanin and betaxanthin content was computed.

RESULTS AND DISCUSSION

It has been reported that in many cases adding appropriate elicitors can significantly increase the yield of secondary metabolites (Zhao *et al*., 18). Such elicitation is recognised as one of the most promising strategies for enhancing betalain production, partly because knowledge of their physiological functions and roles in plant cell defence systems can be exploited. To explore the potential of two biotic elicitors, *viz*., methyl jasmonate (MJ) and β-glucan, in biosynthesis of betalains in callus cultures of bougainvillea cv. Bhabha, the callus were subjected to different levels of MJ and β-glucan.

In the present study, the response coefficient significantly (P≤ 0.05) varied with the supplementation of different MJ levels in the culture medium (Table 1). Significant rapid increase was noticed in response coefficient (81.25%) when MJ was supplemented at 0.5 µM. Higher concentration of MJ (1 µM), however led to decline in response coefficient (67.50%). This suggests that supplementation of 0.5 µM MJ in the culture medium was sufficient to induce betalains in bougainvillea callus. Earliest pigment initiation (7 days) and intensification (13.75 days) was recorded when the medium was supplemented with 0.5 µM MJ. No significant differences in pigment initiation and intensification were observed when the concentration was 0.1 and 0.3 µM. Pigment initiation and intensification was delayed when the media was supplemented with 1 µM MJ. Lower concentrations (0.1, 0.3 and 0.5 µM) efficiently elevated the production of both betacyanins and betaxanthins (Fig.1). Among the concentrations tested, the most effective concentration was 0.5 µM (betacyanin- 0.65 mg/g FW, betaxanthin- 0.48 mg/g FW). The higher concentration (1 µM) was less effective in stimulating the synthesis of betacyanins and betaxanthins.

Thus, MJ acted as a positive inducer of betalain biosynthesis in bougainvillea callus cultures. Methyl jasmonate has been found to increase betacyanin levels in suspension cultures of *Portulaca* sp. cv. Jewel (Bhuyian and Adachi, 2) and hairy root cultures of *B.vulgaris* cv. Ruby (Suresh *et al*., 14). MJ was shown to induce the accumulation of secondary metabolites by turning on the transcripton of several genes involved in their biosynthesis (Chaichana and Dheeranupattana, 4). The enhancement of betacyanins and betaxanthins observed in the present study could be due to the expression of gene(s) encoding enzymes of betalain biosynthesis. Jasmonic acid and MJ have been demonstrated as signal transducers in the intracellular signal cascade that begins with the interaction of an elicitor molecule with the plant cell surface and

Table 1. Effect of methyl jasmonate levels on betalain biosynthesis in bougainvillea callus cultures.

Treatment (µM)	Response coefficient (%)	No. of days taken for pigment	
		Initiation	Intensification
0.0 (control)	62.50	14.50	22.75
0.1	71.25	8.75	16.00
0.3	73.75	8.25	15.25
0.5	81.25	7.00	13.75
1.0	67.50	9.50	17.25
CD at 5%	5.64	1.08	1.42

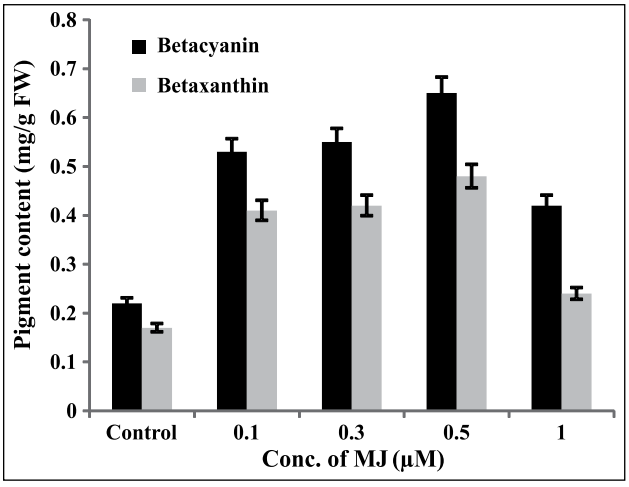

Fig. 1. Effect of methyl jasmonate on *in vitro* betalain biosynthesis.

results ultimately in the accumulation of secondary compounds (Gundlach *et al*., 7).

A statistically significant and positive correlation was found between response coefficient and betacyanin (r = 0.935) and betaxanthin (r = 0.952) content. The strong correlation between these variables indicates that callus cultures with high response coefficient constitute - a good index for ability to produce more betalains.

β-glucan was also effective in stimulating the biosynthesis of betalain pigments in bougainvillea callus cultures (Table 2). In the present study, the concentration of 0.5 mg/l recorded maximum response coefficient (76.25%) which was significantly higher than all other concentrations tested. However, at higher concentration (1 mg/l) there was a decrease in response coefficient. Earliest pigment initiation (9 days) and intensification (16.5 days) was recorded with 0.5 mg/l β-glucan. Though all the treatments were able to induce pigmentation the best results were obtained with 0.5 mg/l concentration (Fig. 2). The betacyanin content was estimated to be 0.35 mg/g FW, while the betaxanthin content was 0.22 mg/g FW under this treatment. A statistically significant and positive correlation was found between response coefficient and betacyanin (r = 0.992) and betaxanthin (r = 0.975) content. Since β-glucan was identified as released elicitors in plants (Yoshikawa *et al*., 17), it has been used to stimulate the accumulation of phytoalexins, such as isoflavonoids, coumarin and lignin. β-glucan was also shown to be effective for anthocyanin production in ohelo suspension culture (Fang *et al*., 6). The results of the present study also confirmed the positive effect of β-glucan on elicitation of betalain pigments in callus cultures of bougainvillea.

Experiments were conducted to explore the potential of three abiotic elicitors, *viz*., $CaCl_2$, FeEDTA and $CuSO_4$ in biosynthesis of betalains in callus cultures of bougainvillea cv. Bhabha. Among the three concentrations of calcium chloride (5, 10 and 15 g/l), maximum response coefficient (78.75%) was observed when the medium was supplemented with

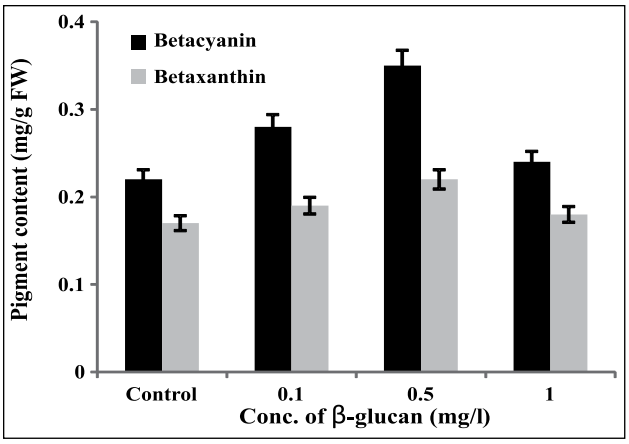

Fig. 2. Effect of β-glucan on *in vitro* betalain biosynthesis.

5 g/l calcium chloride (Table 3). This was significantly higher than all the treatments (P≤ 0.05). This treatment also recorded earliest pigment initiation (8.50 days) and intensification (15.75 days). However, no statistical differences were recorded among the treatments 10 and 15 g/l with respect to response coefficient and number of days required for pigment initiation. Both the content of betacyanin (0.61 mg/g FW) and betaxanthin (0.42 mg/g FW) was significantly higher when calcium chloride was used at 5 g/l (Fig. 3). The pigment content also increased at higher concentrations but it was lower as compared to the above treatment. Similar results were reported by Savitha *et al*. (13) in *B. vulgaris* L. hairy root cultures. A statistically significant and positive correlation was found between response coefficient and betacyanin (r = 0.979) and betaxanthin (r = 0.994) contents. The strong correlation between these variables indicates that callus cultures with high response coefficient constitute a good index forability to produce more betalains.

In the present study, the higher production of betalain pigments over control cultures, irrespective of the concentration of $CaCl_2$ used, could be attributed to its reported role as secondary messenger in signalling

Table 2. Effect of β-glucan levels on betalain biosynthesis in bougainvillea callus cultures.

Treatment (mg/ l)	Response coefficient (%)	No. of days taken for pigment Initiation	Intensification
0.0 (control)	62.50	14.50	22.75
0.1	70.00	10.75	18.25
0.5	76.25	9.00	16.50
1.0	63.75	11.25	19.25
CD at 5%	4.77	1.15	1.03

Table 3. Effect of Ca^{2+} ions on betalain biosynthesis in bougainvillea callus cultures.

Treatment (g/ l)	Response coefficient (%)	No. of days taken for pigment Initiation	Intensification
MS (control)	62.50	14.50	22.75
5	78.75	8.50	15.75
10	72.50	9.75	17.25
15	68.75	10.25	17.50
CD at 5%	4.21	1.23	1.21

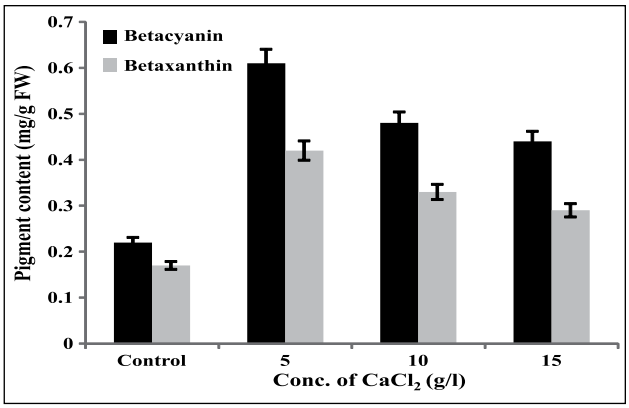

Fig. 3. Effect of Ca^{2+} ions on *in vitro* betalain biosynthesis.

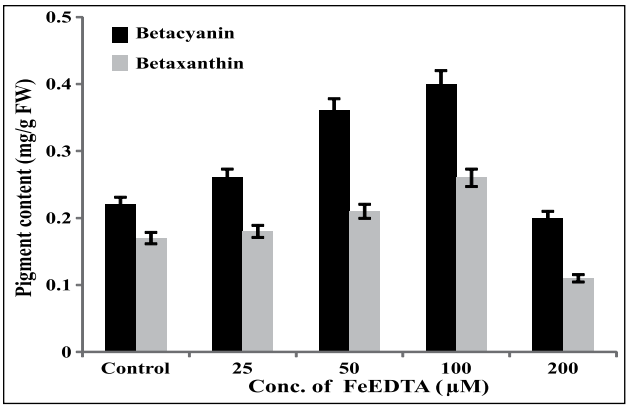

Fig. 4. Effect of Fe^{2+} ions on *in vitro* betalain biosynthesis.

the responses that follow elicitation (Pitta-Alvarez *et al*., 11). The control of intra-cellular free calcium concentration itself could be due to external stimuli that regulate calcium transport within or among the cell organelles or by the introduction of exogenous calcium into the cell (Dauwalder *et al*., 5).

When FeEDTA was used as an abiotic elicitor, maximum response coefficient (77.50%) in cultures was observed at 100 µM concentration which was significantly higher than control (Table 4). Among the different concentrations of FeEDTA tested, earliest response to pigment initiation (9.25 days) was also observed with 100 µM. Pigment intensification took lowest time under this concentration. At higher or lower concentrations more number of days were required for pigment initiation and intensification. Significant increase in the content of betacyanin (0.40 mg/g FW) and betaxanthin (0.26 mg/g FW) was observed under 100 µM FeEDTA (Fig. 4). At lower concentration (25 µM) there was a slight increase in betaxanthin and betacyanin contents. Higher concentration (200 µM) caused decline in both betaxanthin and betacyanin contents. A statistically significant and positive correlation was found between response coefficient and betacyanin (r = 0.957) and betaxanthin (r = 0.979) contents.

Table 4. Effect of Fe^{2+} ions on betalain biosynthesis in bougainvillea callus cultures.

Treatment (µM)	Response coefficient (%)	No. of days taken for pigment	
		Initiation	Intensification
MS (control)	62.50	14.50	22.75
25	66.25	12.25	20.50
50	73.75	10.50	18.75
100	77.50	9.25	17.25
200	53.75	13.75	22.50
CD at 5%	4.91	1.13	1.37

In table beet increase in betalain content was observed when Fe^{2+} was added at an elevated concentration (Akita *et al*., 1). In suspension cultured cells of *Portulaca* maximum increase in betacyanin content was observed when Fe^{2+} was used at a concentration of 100 µM (Bhuyian and Adachi, 2). The results of the present study corroborates with the above findings. Yamamoto *et al*. (16) reported that tyrosine hydroxylase (TOH), an enzyme that is involved with early reaction intermediates of betalain biosynthesis, is activated by the Fe^{2+} ion. Such an activation of TOH enzyme might also influence the bio-synthesis rate of betacyanin pigments. In the present study, the increase in response coefficient, betaxanthin and betacyanin content could be due to the optimum iron availability to the culture medium or could be due to the stimulation of enzymes involved in the biosynthesis of betalains. Therefore, it can be postulated that Fe^{2+} in the form of FeEDTA can be used as an effective elicitor for the production of betalains.

Different concentrations of copper sulphate (10, 20, 40 and 80 µM) were applied to standard MS medium to evaluate its potential to increase the betalain content in callus cultures of bougainvillea cv. Bhabha. Maximum response coefficient (75%) was noted when $CuSO_4$ was used at a concentration of 20 µM in the culture medium which was significantly higher than control. Beyond this concentration there was a decrease in response coefficient (Table 5). Earliest pigment initiation (10 days) and intensification was observed at concentration of 20 µM. At lower or higher concentrations the number of days required for pigment initiation and intensification was more. There are few reports showing the effects of the addition of microelements on the production of betalains. Obrenovic (10) reported a negative effect of an increase of Cu^{2+} on the accumulation of

Table 5. Effect of Cu^{2+} ions on betalain biosynthesis in bougainvillea callus cultures.

Treatment (µM)	Response coefficient (%)	No. of days taken for pigment Initiation	Intensification
MS (control)	62.50	14.50	22.75
10	63.75	12.50	20.75
20	75.00	10.00	18.50
40	71.25	11.50	19.75
80	56.25	13.25	22.25
CD at 5%	4.50	1.35	1.11

betacyanins in callus of *Portulaca grandiflora* and *Amaranthus caudatus* seedlings. In these studies, induction of betacyanin biosynthesis was inhibited by the presence of a divalent copper chelate. This effect was not observed in the present study.

Copper in the form of $CuSO_4$ was previously reported as a positive stimulant of anthocyanin production in ohelo cell cultures (Fang *et al*., 6). Copper ions increased the betalain level in table beet cell culture when added at an elevated concentration (Trejo-Tapia *et al*., 15). In the present study also maximum increase in betacyanin (0.33 mg/g FW) and betaxanthin content (0.21 mg/g FW) were observed when $CuSO_4$ was added at a concentration of 20 µM in the culture medium (Fig. 5).

In the present study, the increase in response coefficient, betacyanin and betaxanthin contents in bougainvillea callus cultures attributed to the increase in activity of some enzymes involved in biosynthesis of betalains, which could also lead to the activation of gene(s) related to enzymes in the cultures. A statistically significant and positive correlation was found between response coefficient and betacyanin (r = 0.968) and betaxanthin (r = 0.994) contents. Thus, Cu^{2+} in the form of $CuSO_4$ at an optimum concentration can be postulated as an effective elicitor in enhancing betalain levels in bougainvillea callus cultures.

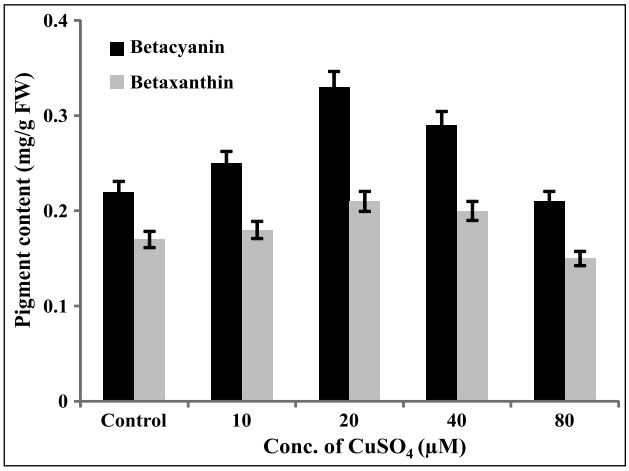

Fig. 5. Effect of Cu^{2+} ions on *in vitro* betalain biosynthesis.

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