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Betalain estimation and callus induction in different explants of *Bougainvillea* spp.

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ABSTRACT

The present study was carried out to investigate the *in vivo* betalain content in bract and leaf of some bougainvillea cultivars and to develop a reliable, rapid and efficient callus induction protocol. The maximum betacyanin content in bracts was recorded in the cv. Bhabha (2.68 mg/g FW) followed by cv. Rao (2.40 mg/g FW). The maximum betaxanthin content in bracts was recorded in the cv. Lady Mary Baring (1.27 mg/g FW). The maximum betacyanin content in leaf was recorded in the cv. Dr H B Singh (0.58 mg/g FW), while the maximum betaxanthin content in leaf was recorded in the cv. Bhabha (0.73 mg/g FW). The total betalain content in bract was estimated to be maximum in the cv. Bhabha (3.60 mg/g FW) followed by cv. Rao (3.36 mg/g FW). The total betalain content in leaf was highest in cv. Bhabha followed by cv. Dr R R Pal. Of the different treatments employed for callus induction on Murashige and Skoog's (MS) medium using leaf explants in bougainvillea cv. Bhabha, the treatment comprising 6 mg/l 2,4-D recorded maximum induction coefficient (98.75%) and minimum days (8.50) was required for callus initiation. In case of internodal explants the induction coefficient was lower and more number of days was required for callus initiation. No callus induction was recorded on bract. Absolutely no callus induction was noted on the MS basal medium devoid of plant hormones (control). The maximum gain in callus biomass accumulation, in both the explants, in terms of fresh and dry cell weight was recorded in the callus cultured on MS medium supplemented with 6 mg/l 2,4-D. This treatment also resulted in lowest fresh and dry cell weight ratio in leaf and internodal explants. Wounding of leaf explants was found to be beneficial for accelerating the callogenesis process. The multiplication of callus was satisfactory on MS medium supplemented with doubled quantity of vitamins and 6 mg/l 2,4-D.

Key words: Betalains, Bougainvillea, Callus, Plant growth regulators

Bougainvillea (*Bougainvillea* spp.), belonging to the dicot family Ncytaginaceae, is one of the most popular ornamental shrubs commonly grown in gardens, porches, boundary walls, lawns and road median steps. The plant has earned a reputation as to be the pride of gardens among the naturalists and ornamentalists due to its wide range of habitats, prolonged flowering season and variety of flower colours. The colour of the bracts is due to the presence of betalains. Betalains, the red-violet betacyanins and the yellow betaxanthins, are a class of water soluble nitrogenous pigments. Betalains accumulate in the cell vacuole of flowers, fruits and leaves of the plants that synthesise them, mainly in epidermal and or sub-epidermal tissues (Jackman and Smith 1996). Betalains reportedly have diverse, desirable activities (Lila 2004), including anti-inflammatory (Lee *et al*. 2006), hepatoprotective (Galati *et al*. 2005), antioxidant (Gentile *et al*. 2004) and chemopreventive activities (Kapadia *et al*. 1996). Recently, it was reported (Sreekanth *et al*. 2007) that betanin induces apoptosis in human chronic mylloid leukemia cells. Hence, betalains are likely to be highly suitable in natural colourants for preparing healthy foods and their consumption is likely to increase.

Biotechnology offers an opportunity to exploit cells, tissues, organs or entire organisms by manipulating them genetically to produce desired compounds and growing them *in vitro* (Rao and Ravishankar 2002). In callus induction, media composition, mainly the hormonal balance, is an important factor that influences *in vitro* culture initiation from explants (Jiang *et al*. 1998). The auxin 2,4-D alone or in combination with cytokinins, is widely used to enhance callus induction and maintenance (Castillo *et al*. 1998). Tissue culture response in ornamentals, which includes callus induction capacity, is influenced by the genotype, explants source, the physiological status of donor plants, the culture medium, and the interaction between them. Rapid and efficient protocol for callus induction can be useful for

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several aims such as establishment of cell suspension culture (Kumar and Kanwar 2007), induction of embryogenic callus (Kulkarni *et al*. 2002), genetic transformation (Frame *et al*. 2000) and secondary metabolites production.There are scanty reports on tissue culture of bougainvillea especially on the callus induction and its subsequent use for the secondary metabolite production. The present work was, undertaken to estimate the betalain content in 42 bougainvillea cultivars and to optimize the tissue culture medium for profuse callus induction from different explants of *Bougainvillea* spp. The comparative effects of PGRs on callus induction were assessed for rapid callus induction, callogenesis coefficient percent and callus growth.

MATERIALS AND METHODS

Betalain estimation in two explants (leaf and bract) of 42 bougainvillea cultivars was carried out based on the method described by Castellanos-Santiago and Yahia (2008) with minor modifications. A known weight of the sample (50 mg) was macerated using double-distilled water. The extracts were centrifuged at 12 000 × g for 10 min in a refrigerated (4°C) centrifuge. Optical density (OD) of the supernatant of each sample was measured at 483 and 535 nm using a UV-vis double-beam spectrophotometer using double-distilled water as blank. The betalain content was calculated according to the given formula:

$$BC\ [mg/g] = [(A(DF)(MW)V/\varepsilon LW)]$$

where, A is the absorption value at the absorption maximum of 535 and 483 nm for betacyanins and betaxanthins, respectively, DF is the dilution factor, V is the volume made up (ml), W is the weight of the sample (g), and L is the path-length (1 cm) of the cuvette. The molecular weight (MW) and molar extinction coefficient (ε) of betanin [MW = 550 g/mol; ε = 60000 l/(mol cm) in H_2O] were applied in order to quantify the betacyanins. Quantitative equivalents of the major betaxanthins were determined by applying the mean molar extinction coefficient [ε = 48 000 l/(mol cm) in H_2O] and molecular weight [MW = 308 g/mol].

For callus induction, the plant material of bougainvillea cultivar Bhabha was collected from the Germplasm Block of International Bougainvillea Germplasm Repository, IARI, New Delhi. Young mature leaves from the middle portion of a healthy and disease-free plant, internodes of 2 mm diameter, and young bracts were selected as explants. The sterilized young leaves were cut into 5 × 5 mm size so that equal portion of lamina was retained on either side of the mid rib. Internodes were cut into small pieces (10 mm long) and individual bracts were separated. Excised leaf segments and other explants were then transferred aseptically and placed horizontally onto the culture medium in the test tube.

Murashige and Skoog's medium supplemented with 30 g/l sucrose was used for callus induction. The ability of seven different treatments, viz. T_0- MS (Control); T_1- MS + 2,4-D (2 mg/l); T_2- MS + 2,4-D (4 mg/l); T_3- MS + 2,4-D (6 mg/l); T_4- MS + 2,4-D (6 mg/l)+ kinetin (6 mg/l); T_5- MS + 2,4-D (1 mg/l) + TDZ (2 mg/l) and T_6- MS + 6-BA (2 mg/l) + NAA (1 mg/l) were analyzed for callus induction. After preparation of culture medium, pH was adjusted to 5.8 ± 0.1 before solidifying with 5.5 g/l agar and autoclaving. The same was then sterilized at 121°C and 15 psi for 16 min and stored for three days before culture of explants. All the cultures were maintained under controlled environment of a culture room at 24 ± 1°C in complete darkness.The best treatment found for callus induction fortified with double the quantity of vitamins was used for callus proliferation. Callus that had been obtained from different treatments were sub-cultured once every three weeks for callus maintenance. At each sub-culture, the calli were selected carefully; brown and dead portions were discarded and healthy calli were excised for sub-culture.

The relative induction of callus was determined as induction coefficient percentage = (total number of induced calli/total number of cultured explants) × 100. Cultures were visually observed daily for callus initiation and observations were recorded on the number of days taken by inoculated explants for callus induction. To assess growth rates of calli, 42-day old callus masses were harvested and fresh and dry cell weight (FCW, DCW) were taken. Fresh cell weight was taken after removing excess of moisture on the surface using blotting paper. Dry cell weight was obtained after drying calli in hot air oven at 45°C for the first 24 h and 55°C for the next 24 h so as to constant the weight. The FCW: DCW ratio was also calculated to obtain the actual gain in callus biomass accumulation. Callus growth status was classified as described by Matkowski (2004). The experiment was laid out in Completely Randomized Design. The data was analyzed by applying the technique of analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Betalain estimation

The data presented in Table 1 shows the results obtained for betalain (betaxanthin and betacyanin) estimation from bract tissue as explants in 42 bougainvillea cultivars. The maximum betacyanin content in bracts was recorded in the cv. Bhabha (2.68 mg/g FW), which was significantly higher than all the other cultivars, followed by cv. Rao (2.40 mg/g FW). The lowest betacyanin content in bracts was recorded in the cv. Shubhra and Hawaiin White.The maximum betaxanthin content in bracts was recorded in the cv. Lady Mary Baring (1.27 mg/g FW), which was significantly higher than all the cultivars, followed by the cv. Singapore Red and Zakairana. The lowest betaxanthin content in bracts was recorded in the cv. Las Banos Beauty. The total betalain content was maximum in the cv. Bhabha (3.60 mg/g FW) which was significantly higher than all the other cultivars studied.

The data presented in Table 2 shows the results obtained for betalain (betaxanthin and betacyanin) estimation using leaf as explant in 42 bougainvillea cultivars. The maximum betacyanin content in leaf was recorded in the cv. Dr H B Singh (0.58 mg/g FW). The lowest betacyanin content in

Table 1 Betalain estimation in bract of bougainvillea cultivars

Cultivar	Betacyanin (mg/g FW)	Betaxanthin (mg/g FW)	Total betalains (mg/g FW)
Aruna	1.80	0.65	2.45
Bhabha	2.68	0.92	3.60
Blondie	0.94	0.84	1.78
Cascade	0.64	0.42	1.06
Chandraberie	1.46	0.60	2.06
Cherry Blossom	0.47	0.21	0.68
Chitra	0.55	0.32	0.88
Dr. H. B. Singh	0.39	0.45	0.84
Dr. R. R. Pal	1.19	0.73	1.91
Elizabeth Agnis	2.14	0.93	3.07
Filoman	1.54	0.48	2.02
Flame	1.25	0.70	1.95
Golden Glow	0.64	0.32	0.96
Hawaiin White	0.21	0.23	0.44
Jayalaxmi	0.92	0.47	1.39
Lady Mary Baring	0.55	1.27	1.82
Las Banos Beauty	0.46	0.16	0.62
Mahara	1.44	0.79	2.23
Mahatma Gandhi	0.79	0.50	1.29
Mary Palmer Special	0.49	0.52	1.01
MatajiAgnihotri	0.77	0.51	1.27
Parthasarthy	0.81	0.90	1.70
Partha	1.22	0.69	1.91
Pink Beauty	0.63	0.25	0.88
Poultoni Special	0.63	0.46	1.09
Radha	1.02	0.32	1.34
Rao	2.40	0.96	3.36
Red September	1.56	0.73	2.29
Refulgen	0.53	0.42	0.95
Rooseville Delight	0.47	0.60	1.07
Shubhra	0.21	0.35	0.56
Singapore Red	1.59	1.07	2.65
Sonnet	1.56	0.90	2.46
Spring Festival	1.22	0.94	2.16
Stanza	0.96	0.94	1.90
Superba	1.20	0.71	1.91
Sweet Heart	0.99	0.57	1.55
Thimma	0.68	0.49	1.17
Torch Glow	0.39	0.25	0.64
Vishakha	1.16	0.42	1.58
Versicolor	1.30	0.97	2.27
Zakairana	0.77	1.05	1.82
CD (P=0.05)	0.10	0.09	0.18

Table 2 Betalain estimation in leaf of bougainvillea cultivars

Cultivar	Betacyanin (mg/g FW)	Betaxanthin (mg/g FW)	Total betalains (mg/g FW)
Aruna	0.23	0.28	0.53
Bhabha	0.48	0.73	1.21
Blondie	0.35	0.49	0.84
Cascade	0.21	0.20	0.41
Chandraberie	0.44	0.66	1.10
Cherry Blossom	0.39	0.46	0.85
Chitra	0.21	0.34	0.56
Dr. H. B. Singh	0.58	0.52	1.10
Dr. R. R. Pal	0.56	0.58	1.13
Elizabeth Agnis	0.30	0.28	0.58
Filoman	0.47	0.44	0.91
Flame	0.28	0.26	0.55
Golden Glow	0.19	0.19	0.38
Hawaiin White	0.35	0.52	0.87
Jayalaxmi	0.22	0.19	0.41
Lady Mary Baring	0.30	0.48	0.78
Las Banos Beauty	0.34	0.52	0.87
Mahara	0.44	0.60	1.04
Mahatma Gandhi	0.20	0.22	0.42
Mary Palmer Special	0.46	0.59	1.05
Mataji Agnihotri	0.21	0.23	0.44
Parthasarthy	0.21	0.31	0.52
Partha	0.37	0.50	0.87
Pink Beauty	0.05	0.09	0.15
Poultoni Special	0.17	0.20	0.37
Radha	0.38	0.36	0.74
Rao	0.34	0.54	0.88
Red September	0.28	0.47	0.75
Refulgen	0.38	0.62	1.00
Rooseville Delight	0.40	0.51	0.91
Shubhra	0.34	0.53	0.87
Singapore Red	0.31	0.44	0.75
Sonnet	0.22	0.30	0.52
Spring Festival	0.37	0.52	0.89
Stanza	0.23	0.37	0.60
Superba	0.27	0.41	0.68
Sweet Heart	0.27	0.26	0.53
Thimma	0.28	0.45	0.73
Torch Glow	0.19	0.26	0.45
Vishakha	0.21	0.34	0.55
Versicolor	0.24	0.34	0.58
Zakairana	0.36	0.53	0.89
CD (P=0.05)	0.06	0.07	0.11

leaf was recorded in the cv. Pink Beauty. The maximum betaxanthin content in leaf was recorded in the cv. Bhabha (0.73 mg/g FW), which was significantly higher than all the other cultivars. The lowest betaxanthin content in leaf was recorded in the cv. Pink Beauty. The total betalain content in leaf was maximum in the cv. Bhabha (1.21 mg/g FW), followed by the cv. Dr R R Pal (1.13 mg/g FW).

Callogenesis

For callus induction, the cultivar Bhabha was chosen. Callus induction experiment was carried out using leaf, bract and internode as explants. No callus induction was recorded when bract used as explant.Wounding of leaf explants had a notable effect on callogenesis. Wounded leaf segments showed high callusing not only on the outer cut surfaces but also on the inner punctured places. Better morphogenic responses in wounded leaflets were also described in other plant species (D' Onofrio and Morini 2003, Pacheco *et al*. 2008). This may be a reflection of different endogenous hormonal levels and cell differentiation levels in tissue segments (Koroch *et al*. 2002).

Vigorous callus induction was recorded in the leaf explants cultured on the MS medium supplemented with 6 mg/l 2,4-D than the other treatments (Table 3). The induced calli did not exhibit any morphological variation. As the incubation duration increased (21-day), calli progressively increased in volume and gradually covered almost entire explants. After 21 day of callus induction, there was an apparent increase in callus weight on MS + 6 mg/l 2,4-D and growth of the callus was more vigorous than that of newly induced callus. It is assumed that the cell proliferation that started at the injured part of leaf segment, may have been due to the accumulation of auxin at the point of injury, which stimulated cell proliferation in the presence of PGRs and their derivatives. The results are consistent with a hypothesis which postulates that the accumulation of auxins at the point of injury stimulated cell proliferation (Ahmad *et al*. 2010). Visually, there were differences worth mentioning in the callus induction and growth among the different treatments after 21-day of culture. For further maintenance of viable calli, these were transferred from induction to multiplication medium, after 27-day of incubation. In case of internodal explants also callus induction was maximum with 6 mg/l 2,4-D (Table 4). But the response coefficient was lower compared to leaf explants and also more number of days was required for callus induction.

Callus induction efficiency

It is well known that, a complete tissue culture protocol needs to be established for each species in terms of nutrient medium, temperature, photoperiod and importantly, concentration of PGRs in the culture medium. Growth regulators, as one kind of signal molecule have been shown to play an important role during the callogenesis (Dodeman *et al*. 1997, Sugiyama and Imamura 2006). The coefficient for callogenesis was recorded approximately in the range of 0 to 98.75% in leaf explants, depending on auxin type, concentration and their combination with cytokinins. Leaf segments cultured on MS medium containing 6 mg/l 2,4-D formed early and more callus than other treatments. The treatment 6 mg/l 2,4-D gave rise to luxuriantly growing callus with the highest induction coefficient (98.75%) and took minimum days (8.50) for callus induction. This was statistically different with the other treatment combinations tried. Hormone-free medium did not induce any callogenic response in leaf explants. Other treatments except control also induced callus formation in cultured leaf segments. As the concentration of 2,4-D in the culture medium increased, callus formation was also increased.

In case of internodal explants, coefficient for callogenesis was recorded approximately in the range of 0 to 88.75% of explants, depending on auxin type, concentration and their combination with cytokinins. Internodal segments cultured on MS medium containing 6 mg/l 2,4-D formed early and more callus than other treatments. The treatment 2,4-D at 6 g/l recorded highest induction coefficient (88.75%) and took minimum days (10.50) for callus induction. Hormone-free medium did not induce any callogenic response in internodal explants.

Callus induction is usually promoted by auxin only (Yang *et al*. 2009, Mousavi *et al*. 2012); however others found that auxin-cytokinin combination were much

Table 3 Effect of PGRs on callus induction in leaf explants of bougainvillea cv. Bhabha

Treatment	Induction coefficient (%)	Days for callusing	Callus fresh weight (mg)	Callus dry weight (mg)	FCW:DCW ratio	Growth status*
T_0	0.00	0.00	0.00	0.00	0.00	-
T_1	52.50	13.50	353.21	67.31	5.25	I
T_2	81.25	11.75	361.45	74.34	4.86	II
T_3	98.75	8.50	386.39	122.87	3.15	IV
T_4	92.50	10.25	370.12	86.19	4.30	III
T_5	86.25	11.25	364.57	76.25	4.78	II
T_6	93.75	10.00	375.48	89.54	4.20	III
CD (P=0.05)	4.27	1.22	5.16	3.64	0.10	

*Growth status (Matkowski 2004): (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.

Table 4 Effect of PGRs on callus induction in internodal explants of bougainvillea cv. Bhabha

Treatment	Induction coefficient (%)	Days for callusing	Callus fresh weight (mg)	Callus dry weight (mg)	FCW:DCW ratio	Growth status*
T_0	0.00	0.00	0.00	0.00	0.00	-
T_1	41.25	15.50	286.81	47.73	6.01	I
T_2	73.75	14.00	300.09	53.57	5.60	II
T_3	88.75	10.50	328.94	85.40	3.85	IV
T_4	80.00	12.75	307.17	60.99	5.04	III
T_5	77.50	13.25	295.05	53.66	5.50	II
T_6	82.50	12.25	311.07	62.86	4.95	III
CD (P=0.05)	4.64	1.34	3.73	1.60	0.10	

*Growth status (Matkowski 2004).

important for callus induction in some plant species (Dhar and Joshi 2005, Lim *et al*. 2009). It is assumed that such variations may be attributed to the type and species of plant, endogenous hormonal balance and explant type. The suitable PGRs for callus induction vary among plant species. In the present study, superior, healthy and vigorous callus with high induction response was recorded with auxin only.

Callus growth and biomass accumulation

With regard to growth status of callus culture on MS medium supplemented with different treatments, only 6 mg/l 2,4-D recorded the type-IV callus. To determine the callus growth in terms of biomass accumulation, fresh cell weight (FCW) at harvest, dry cell weight (DCW) after drying and weight ratio (FCW: DCW) were measured, after 42-day of culture. The fresh and dry cell weights were significantly affected by the different concentrations and combinations of tested PGRs in the culture medium. The leaf derived calli showed a steady growth. Tables 3 and 4 showed that the treatment 6 mg/l 2,4-D resulted in a high cell dry biomass production in both the explants. This treatment had a significant effect on fresh- as well as dry-cell weight and differed significantly with the other tested treatments. The treatment 2 mg/l 2,4-D had less effect on enhancing cell biomass accumulation compared to other levels tested. Duangporn and Siripong (2009) found that 2,4-D had more effect on callus growth individually when compared to NAA.

Not withstanding only the fresh- and dry-cell weight, the ratio of fresh- to dry-cell weight (FCW: DCW), an index of cell water content, was also calculated to know the actual gain in callus biomass accumulation. The callus with high relative dry weight has lower infux of water. Between the different concentrations and combinations of PGRs tried in leaf explants, treatment 6 mg/l 2,4-D resulted in the lowest FCW: DCW ratio (3.15) which indicated that the callus produced was not watery and gained better biomass accumulation. Further, ininternodal explants also 6 mg/l 2,4-D resulted in lowest FCW: DCW ratio. Explants cultured on MS medium without PGRs did not induce any callus; so there was no fresh- and dry-cell weight value for this treatment.

Callus maintenance

The explant and treatment which performed better for callus induction was further modified to get sufficient quantity of callus during long-term culture. In this regard, MS medium supplemented with 6 mg/l 2,4-D was altered to some extent and optimized for encouraging and maintaining the callus growth from leaf explants. The viable calli were transferred from induction to maintenance medium, after 27-days of incubation. After the first sub-culture, the disintegrated mother leaf explant was removed from the calli otherwise it not only created the browning problem in surrounding callus tissues but also released the phenolics, resulting in browning of the culture medium. As soon as the disintegrated mother leaf explant was removed from the callus, their growth was slowed down and appeared not much healthy. To counteract this effect, quantity of vitamins of the MS basal medium was doubled, restoring higher callus proliferation. This may be due to the fact that the calli were getting some essential growth regulating substances from the mother leaf explants tissue. The maintenance of bougainvillea callus from leaf explants provides much convenient way for further studies in which bougainvillea callus could be widely used with sustained growth.

The present investigation reports an efficient, simple and easy-to-handle protocol for betalain estimation, callus induction and multiplication from different explants of *Bougainvillea* spp. Leaf explants are ideal for rapid callus induction and proliferation in bougainvillea and 2,4-D (6 mg/l) is much efficient in callus induction and biomass accumulation.

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