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ISOLATION OF CALLUS LINES OF *CELOSIA CRISTATA* L. WITH VARIATION IN BETALAIN CONTENT

MRUNAL I. WARHADE AND RUPESH S. BADERE*

Department of Botany, Hislop College, Temple Road, Civil Lines, Nagpur- 440 001 (India)

**Email: rsbadere@rediffmail.com*

The present paper reports the isolation of three callus lines of cockscomb (*Celosia cristata* L.) varying in colour viz. magenta, red and dark-red. The calli were induced from shoot-tip explants harvested from 4, 8 and 12 days old seedlings. NAA or 2,4-D could induce callusing only in presence of cytokinins like BAP and kinetin. The total betalain content of dark-red callus was found to be about twice than that of magenta and red coloured callus. Similarly, the content of constituent pigments viz. amaranthine, betanin and betaxanthin was also found to be more than twice in dark-red callus as compared to magenta and red callus. Similarly, the betanin content in red coloured callus was fifty percent higher than that of magenta coloured callus. In contrast to this the content of betalamic acid was found to be almost equal in the three callus lines.

Keywords: betacyanin, betaxanthin, callus culture, cockscomb, somaclonal variation

Celosia cristata L., commonly known as cockscomb belongs to family Amarantheceae. It is widely spread in subtropical and temperate zones of Africa, South America and South-east Asia. The plant is mostly used for landscaping and avenue plantation due to its attractive colour variation from yellow to various shades of red and violet (Schliemann 2001, Taha *et al*. 2012). The varied colours of cockscomb are due to presence of betalains. These are water soluble immonium conjugates of betalamic acid with cyclo-DOPA and amino acids or amines. These pigments are mostly present in species of the order Caryophyllales which comprise the violet betacyanins and yellow betaxanthins red (Strack *et al*. 2003).

Since ancient times many of the pigments are being used for dyeing cloths, printing, painting, food colouring and as cult items in sacred ceremonies (Georgiev *et al.* 2008). The Aztecs used leaves of red-violet amaranths to symbolize human blood during sacrificial ceremonies (Zryd and Christinet 2004). Betalains are being used as natural colourant since centuries but their importance as food colourant increased recently due to their high antioxidant activities and use as the alternatives for synthetic food colourants. Betalains also has desirable clinical actions like anti-inflammatory, hepatoprotective, cancer chemo-preventative, antioxidant, protecting low density lipoproteins (LDLs) from oxidation (Georgiev *et al*. 2008).

Thus, looking at the varied activities of betalains and *C. cristata* being its rich source, it was decided to attempt the production of betalains *in vitro* from the explants of *C. cristata*. Such approaches have been successful in recent past in case of *Celosia argentea* (Schliemann *et al*. 2001), *Amaranthus tricolor* (Biswas *et al*. 2013), *Portulaca* sp. (Kishima *et al*. 1991), *Chenopodium rubrum* (Berlin *et al*. 1986), *A. spinosa* (Stintzing *et al*. 2004), *Beta vulgaris* (Leathers *et al*. 1992) and *Phytolacca americana* (Schliemann *et al*. 1996).

MATERIALS AND METHODS

Collection and Surface Sterilization of Seeds

Seeds of *C. cristata* were obtained from Satpuda Botanic Garden, Nagpur (India) on gratis. The seeds were surface sterilized using sodium hypochlorite, mercuric chloride and 70% ethanol as described by Warhade and Badere (2014). Surface sterilized seeds were allowed to germinate under aseptic conditions by inoculating them over filter paper moistened with sterile distilled water in a flask. The flasks were incubated at 25± 2° C and 16h photoperiod.

Harvesting of explants

The explants like shoot tip, hypocotyl and cotyledonary leaf were harvested from 4, 8, and 12d-old seedlings and surface sterilized as described by Warhade and Badere (2014). Subsequently, twenty-five explants each were

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inoculated over MS medium (Murashige and Skoog 1962) supplemented with PGRs like BAP, kinetin, NAA, 2,4-D and 2,4,5-T either singly or in combination. The cultures were maintained at 25± 2° C and 16h photoperiod.

Raising and Maintenance of Calli

Some of explants inoculated over the medium formed calli. Amongst these, certain calli showed sectors of various colours. Such coloured sectors were only subcultured over the same medium after three to four weeks. With few subcultures the calli with uniform colour were formed. During the initial sub-cultures calli remained compact. However later on, friability of callus increased. The calli exhibiting stable pigmentation were maintained for upto 3 years by sub-culturing them over the same medium regularly.

Selection of Callus Lines

The calli with intense and uniform pigmentation were selected for thin layer chromatography (TLC) analysis according to Harborne (1998). 1g each of fresh callus and inflorescence of *C. cristata* (as a control) was extracted in 2ml of ice cold 1% methanolic hydrochloric acid. This extract was centrifuged at 10,000rpm for 12min. at 4° C. Supernatant was collected and allowed to evaporate till the total volume of extract was reduced to half. The reduced extract was applied on to silica gel G TLC plate. The spots were resolved using top layer of BAW (n-butanol (4) : Glacial acetic acid (1) : distilled water (5)) solvent system. The complete procedure was carried under dim light.

Quantification of Betalains

In the next phase, calli giving satisfactory TLC profile were selected for quantification of betalains. For this the calli were extracted in 5 volumes of 1% methanolic hydrochloric acid and centrifuged at 10,000 rpm for 12min. Supernatant was used for photometric quantification of betalains. The absorbance of extract was recorded at 538, 536, 430 and 480nm against 1% methanolic hydrochloric acid as blank.

Experiments were performed in triplicate and the betalain content was calculated according to Cai *et al.* (1998) with a slight modification as per the following formula:

$$\text{Pigment content (mg/g fresh weight)} = A \times M \times V_a \times D \ \frac{100}{10 \times \varepsilon \times L \times W_a}$$

Where, A is the absorption at given wavelength, M is molecular weight of the pigment, D is dilution factor, Va is total volume of fresh pulp solution (ml), Wa is fresh weight of pulp solution, ε is molecular extinction coefficient of the pigment and L is path length.

$$\text{Pigment contribution} = \frac{\text{Quantity of a pigment}}{\text{Total betalain content}}$$

$$\text{Pigment ratio} = \frac{\text{Quantity of one pigment}}{\text{Quantity of another pigment}}$$

The λ_{max}, molecular extinction coefficient and molecular weight of each pigment is given in the table-1

RESULTS

Callus induction in *C. cristata* was found to be affected by various factors like age of explant,

Table 1. Molecular characteristics of different betalain pigments.

SN	Pigment	λ_{max} (nm)	Molecular extinction coefficient (L/mol cm)	Molecular weight (g/mol)	Reference
1	Amaranthine	536	5.66×10^4	726.6	Cai *et al.*, 1998
2	Betanin	538	6.00×10^4	550	Castellanos-Santiago and Yahia, 2008
3	Betalamic acid	430	2.40×10^4	212	Biswas *et al.*, 2013
4	Betaxanthin	480	48.00×10^6	309	Girod and Zryd, 1991

Table 2. Induction of callus from shoot-tip explants derived from seedlings of cockscomb over MS media fortified with NAA, BAP & Kinetin.

Plant growth regulator (µM)			Age of explant		
			4 day	8 day	12 day
NAA	BAP	Kn	Freq. (%)	Freq. (%)	Freq. (%)
5.0	**-**	**-**	0	0	0
10.0	**-**	**-**	0	0	0
15.0	**-**	**-**	0	0	0
20.0	**-**	**-**	0	0	0
5.0	**0.44**	**-**	0	20 (RW)	0
10.0	**0.44**	**-**	0	28 (RW)	0
15.0	**0.44**	**-**	0	20 (RW)	16 (G)
20.0	**0.44**	**-**	0	24 (RW)	0
5.0	**0.88**	**-**	0	12 (RW)	12 (RW)
10.0	**0.88**	**-**	0	20 (RW)	12 (RW)
15.0	**0.88**	**-**	0	20 (RW)	0
20.0	**0.88**	**-**	0	8 (RW)	8 (RW)
5.0	**-**	**0.46**	0	12 (Y)	4 (Y)
10.0	**-**	**0.46**	0	0	8 (Y)
15.0	**-**	**0.46**	0	8 (Y)	0
20.0	**-**	**0.46**	0	0	0
5.0	**-**	**0.92**	0	4 (Y)	0
10.0	**-**	**0.92**	0	4 (RW)	0
15.0	**-**	**0.92**	0	4 (RW)	0
20.0	**-**	**0.92**	0	0	0

Note: 1) Hypocotyl and Cotyledonary leaf gave no response.

2) Data collected after 20 days of inoculation.

RW = Reddish-white, G = Green, Y = Yellowish

Table 3. Induction of callus from shoot-tip explants derived from seedlings of cockscomb over MS media fortified with 2,4-D, BAP & Kinetin.

Plant growth regulator (µM)			Age of explants		
			4 day	8 day	12 day
2,4-D	BAP	Kn	Freq. (%)	Freq. (%)	Freq. (%)
4.5	**-**	**-**	0	0	0
9.0	**-**	**-**	0	0	0
13.5	**-**	**-**	0	0	0
17.0	**-**	**-**	0	0	0
4.5	**0.44**	**-**	16 (RY)	0	20 (RY)
9.0	**0.44**	**-**	0	0	8 (RY)
13.5	**0.44**	**-**	0	0	44 (RY)
17.0	**0.44**	**-**	0	0	20 (RY)
4.5	**0.88**	**-**	16 (G)	0	36 (R)
9.0	**0.88**	**-**	8 (G)	8 (R)	16 (R)
13.5	**0.88**	**-**	0	0	12 (Y)
17.0	**0.88**	**-**	0	0	4 (Y)
4.5	**-**	**0.46**	12 (G)	0	0
9.0	**-**	**0.46**	20 (G)	0	0
13.5	**-**	**0.46**	0	0	0
17.0	**-**	**0.46**	0	0	0
4.5	**-**	**0.92**	20 (G)	0	0
9.0	**-**	**0.92**	12 (G)	0	0
13.5	**-**	**0.92**	0	0	0
17.0	**-**	**0.92**	0	0	0

Note: 1) Hypocotyl, Cotyledonary leaf gave no response

2) Data collected after 20 days of inoculation

RY = Reddish-yellow, R = Red, G = Green, Y = Yellowish

its source, PGRs etc. The response varied in terms of frequency, friability of callus and its colour. To determine the suitable age and source of explant and effective concentration of PGRs to induce callus; explants like shoot tip, hypocotyl and cotyledonary leaf were harvested from 4, 8 and 12d-old seedlings. These explants were then inoculated over MS medium containing BAP, kinetin, 2,4-D, NAA and TCA either alone or in combination at various concentrations. Amongst all explants, only shoot tip explant formed callus while the others did not give any response. NAA and 2,4-D alone were unable to induce callus (Table 2 & 3). Although, 2,4,5-T alone induced callus but the frequency was rather low (Table 4). Presence of cytokinins was necessary for induction of callus in this case. However, out of the two cytokinins tested, viz. BAP and kinetin, BAP proved to be more effective in callus formation in most of the cases, particularly, in combination with NAA (Table 2-4). As far as the age of explants is concerned, 4d-old explants were not much responsive to PGRs in terms of callusing. In case of NAA, 8d-old explants responded better than the older explants (Table 2), whereas in case of 2,4-D 12-d old explants were more responsive than the younger explants (Table 3).

Table 4. Induction of callus from shoot-tip explants derived from seedlings of cockscomb over MS media fortified with TCA, BAP & Kinetin.

Growth regulators (µM)			Age of explants		
			4 day	8 day	12 day
TCA	BAP	Kn	Freq. (%)	Freq. (%)	Freq. (%)
3.9	**-**	**-**	0	4 (RG)	28 (Y)
7.8	**-**	**-**	0	0	16 (Y)
11.7	**-**	**-**	0	8 (RG)	4 (Y)
15.6	**-**	**-**	0	16 (RG)	0
3.9	**0.44**	**-**	0	4 (G)	36 (Y)
7.8	**0.44**	**-**	0	0	8 (Y)
11.7	**0.44**	**-**	0	0	0
15.6	**0.44**	**-**	0	0	0
3.9	**0.88**	**-**	4 (G)	8 (G)	0
7.8	**0.88**	**-**	16 (G)	4 (G)	0
11.7	**0.88**	**-**	8 (G)	0	12 (G)
15.6	**0.88**	**-**	0	0	0
3.9	**-**	**0.46**	0	0	8 (G)
7.8	**-**	**0.46**	0	0	0
11.7	**-**	**0.46**	0	4 (G)	0
15.6	**-**	**0.46**	0	0	8 (G)
3.9	**-**	**0.92**	0	4 (G)	0
7.8	**-**	**0.92**	0	4 (G)	0
11.7	**-**	**0.92**	0	4 (G)	0
15.6	**-**	**0.92**	0	4 (G)	8 (G)

Note: 1) Hypocotyl, Cotyledonary leaf gave no response

2) Data collected after 20 days of inoculation

G = Green, Y = Yellowish, RG = Reddish green

Table 6. Pigment contribution of the callus lines.

Callus line	Amaranthin	Betanin	Betalamic acid	Betaxanthin
Magenta	0.42	0.30	0.12	0.16
Red	0.47	0.33	0.09	0.11
Dark-red	0.50	0.35	0.05	0.10

Table 7. Pigment ratio in the callus lines.

	Amaranthin	Betanin	Betalamic acid	Betaxanthin
		Magenta callus line		
Amaranthin	1.00	6.09	25.88	20.70
Betanin		1.00	4.25	3.40
Betalamic acid			1.00	8.00
Betaxanthin				1.00
		Red callus line		
Amaranthin	1.00	1.42	5.22	4.27
Betanin		1.00	3.61	3.00
Betalamic acid			1.00	0.82
Betaxanthin				1.00
		Dark-red callus line		
Amaranthin	1. 00	1.43	10.00	5.00
Betanin		1.00	7.00	3.50
Betalamic acid			1.00	0.50
Betaxanthin				1.00

Table 5. Quantification of betalain pigments in the callus lines.

Callus line	Average quantity (mg/g of fresh weight)				
	Amaranthin	Betanin	Betalamic acid	Betaxanthin	Total*
Magenta	4.64 ± 0.348	3.25 ± 0.245	0.74 ± 0.015	0.99 ± 0.074	9.62
Red	5.48 ± 0.043	3.84 ± 0.029	1.00 ± 0.015	1.28 ± 0.049	11.60
Dark-red	15.08 ± 0.271	10.58 ± 0.171	1.36 ± 0.028	2.88 ± 0.063	29.90

* Calculated sum total of all the pigments.

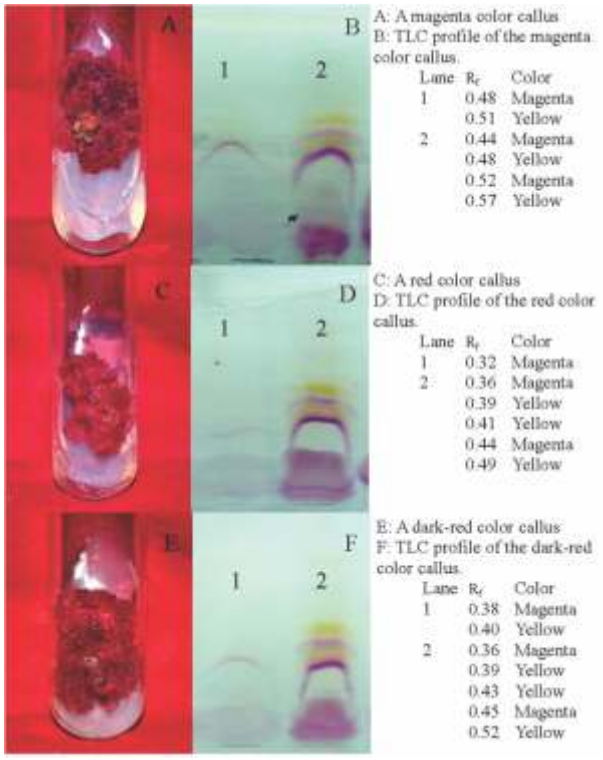

Figure.1: The profile of betalain of calli induced over MS medium containing 2, 4-D 13.5µM+ BAP 0.44µM (Lane 1) and the inflorescence (Lane 2).

Calli with sectors of six different colours/shades viz. yellowish, green, reddish-green, reddish-white, reddish-yellow and red were induced by the various PGR combinations. NAA in combination with BAP induced green and reddish-white calli, while in combination with kinetin it induced yellowish and reddish-white calli. Similarly, 2,4-D in combination with BAP induced yellowish, green, reddish-yellow and red calli, and in combination with kinetin induced only green calli. On the other hand, irrespective of cytokinins, 2,4,5-Trichlorophenoxyacetic acid induced yellowish, green and reddish-green calli (Table 2-4).

Initially, all the calli were more or less compact. Amongst these calli, the calli with red sectors induced by 13.5µM 2,4-D with 0.44µM BAP were selected and subcultured over the same medium. Later on, with the subculturing for 3-5 generation the calli became friable. Amongst these sub-cultures, calli lines with different shades i.e. magenta, red and dark-red were isolated and were qualitatively and quantitatively analyzed for betalain content. The qualitative analysis was performed by silica gel G TLC. Over TLC the extract of inflorescence (used as a control) displayed 4-5 bands of either magenta or yellow colour. However, the numbers of bands displayed by the calli were either 1 or 2 (Fig. 1).

The content of betalains viz. amaranthin, betanin, betalamic acid and betaxanthin were determined spectrophotometrically in the three lines of calli. In general, quantity of all the pigments except betalamic acid, were significantly higher in dark-red calli. Particularly in dark-red calli, amaranthin and betanin were appreciably higher than the magenta or red calli. The levels of betalamic acid, however, were more or less same in all the three callus lines (Table 5).

Mere comparison of quantity of various pigments was not of much help to derive any conclusion (Table 5). Hence, we calculated the ratio of each pigment to the total quantity of betalains and the mutual ratio of the pigments. The ratio of a particular pigment to the total quantity of betalains was termed as 'pigment contribution'. Although, minor differences existed in terms of pigment contribution in the three lines, but that of betalamic acid was clearly apparent in these lines. The pigment contribiution of betalamic acid was highest in magenta calli followed by red and dark-red in that order (Table 6).

We also calculated the ratio of the two betalain pigments in relation to each other. This was termed as 'pigment ratio'. We found that amaranthin-betanin, amaranthin-betalamic acid and amaranthin-betaxanthin ratio was appreciably high in magenta callus line as compared to red and dark-red callus lines. In the same manner betalin-betalmic acid ratio was comparatively higher in dark-red calli when compared with the magenta and red calli. We also found that betalamic acid-betaxanthin ratio was maximum in magent calli as compared to the other two callus lines (Table 7).

DISCUSSION

Out of all the explants used, only shoot tips could be induced to callogenesis under the conditions tested. Although, Rao *et al*. (2005) are view that all explants can be induced to form calli but Sharma *et al*. (2013) found shoot tip explants to be more amenable to callusing than cotyledonary leaf and hypocotyls. Moreover, they found that cytokinins, in small concentration, play a synergistic role with auxins and induce the callus. The same observations were also recorded in the present study. While NAA and 2,4-D alone could not induce callus; presence of cytokinins like BAP or kinetin helped the explant to develop callus over the medium. The combination of 2,4-D with BAP was found to be more effective in callus induction as compared to other combinations. Similar results were reported in *Alternanthera sessilis* (Das and Borua 2014), *Simmondsia chinensis* (Kumar *et al.* 2013), *Saueda nudiflora* (Singh *et al*. 2004). However, in contrast to these results, 2,4-D alone or its combination with kinetin was more effective in *Gymnema sylvestris* (Roy *et al*. 2008) and *Aerva lanata* (Surya *et al*. 2012). Unlike NAA and 2,4-D; 2,4,5-T alone induced callus from the shoot tip explant. Above that in combination with BAP or kinetin frequency of calli induction decreased. Similar response has been made in *Dactylis glomerata* (Atis *et al*. 2013), Rose var. Livin Easy (Estabrooks *et al*. 2007) and *Cicer arietinum* (Sagare *et al*. 1993).

Three callus lines, designated as magenta, red and dark-red, differing in the terms of their colour shades were isolated in the present investigation. As expected, the total betalain content varied in these lines, particularly dark-red calli were rich in betalains as compared to the other two. Betalains are derived from L-tyrosine via L-DOPA and/or cyclo-DOPA. The aglycone betanidin or its precursor cyclo-DOPA is acylated or glycosylated to form various betacyanins. In the biosynthetic pathway of betacyanins and betaxanthins, betalamic acid is a central intermediate. The pigments like amaranthin, betanin and betaxanthins are formed due to condensation of betalamic acid with their precursors. For example Cyclo-DOPA-5-O-Glucoside condenses with betalamic acid to form betanin. Similarly, amaranthin is the condensation product of betalamic acid and Cyclo-DOPA Glucuronylglucoside and betaxanthin is formed from the condensation of betalamic acid with 3-Methoxytyramine (Strack *et al*. 2003). In the present investigation the content of betalamic acid in all the callus lines was nearly equal. However, the other pigments varied significantly in these cell lines and increased from magenta to red and to dark-red calli. Keeping in view the role of central intermediate being played by betalamic acid during biosynthesis of betalains, it could be said that the content of betalamic acid was minimum and invariable in the callus lines as it was being consumed during the synthesis of other betalain derivatives. It can be undoubtedly said that the three callus lines viz. magenta, red and dark-red differed only because of accumulation of differential quantity of betalains with minimum in magenta and maximum in dark-red. This denotes the increased ability of pigment biosynthesis of one line over the others. This increased ability may be due to either more availability of precursor and/or increased enzyme activity. Moreover, it also indicates the higher efficiency of these lines in storing betalains in their vacuoles (Tanaka *et al*. 2008). Whatsoever, this clearly indicates the differential gene expression between the lines. It is well known that genetic variation is induced during callus formation under the influence of exogenous plant growth regulators (Wang *et al*. 2011). Hence, a callus may develop sectors which might be genetically different from each other showing altogether different patterns of gene expression. In our case, this differential pattern was apparent in form of various callus lines differing in their shades. Earlier to this similar observations have been reported in *Portulaca* sp. (Noda and Adachi 2000), *Beta vulgaris* var. Detroit dark red (Akita *et al*. 2000) and *Chenopodium rubrum* (Berlin *et al.* 1986).

Thus, our attempt to select somaclonal variants induced during callogenesis resulted in the isolation of three callus lines differing in their betalain content. The dark-red callus line, which was found to accumulate higher quantity of betalains can be further used to raise suspension cultures for further studies.

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