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The in vitro production of an anthocyanin from callus cultures of *Oxalis linearis*

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Abstract

Callus growth and the production of anthocyanins were sustained on the salts and vitamins of Murashige and Skoog. Callus growth was stimulated at a concentration of 8–32 μM α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Benzyladenine (BA) and zeatin at 8 μM inhibited callus growth whereas isopentenyladenine (iP) stimulated callus growth. NAA repressed anthocyanin production with an increase in NAA from 8–32 μM. Anthocyanin synthesis was promoted by an increase in 2,4-D from 0.5 to 2 μM and decreased thereafter up to a concentration 32 μM 2,4-D. A concentration of 8 μM BA, thidiazuron and zeatin, respectively stimulated pigment production. Sucrose stimulated callus growth at 60 mM and pigment production at 120–360 mM.

Abbreviations: BA – 6-benzyladenine, 2,4-D – 2,4-dichlorophenoxyacetic acid, NAA – α-naphthaleneacetic acid, iP – isopentenyladenine, TZ – thidiazuron – N-phenyl-N′-1,2,3-thiadiazol-5-yl-urea, Bu-HCl – Butanol-2N HCl, BAW – Butanol-acetic acid-water

Introduction

Anthocyanins are currently studied anew as a source of colorants due to public pressure against the use of synthetic dyes. The small biomass produced by members of the Oxalidaceae does not make it economically feasible to produce pigments on a large scale from plants. Recently cyanidin-3-glucoside was isolated from in vitro cultured *Oxalis linearis* Jacq. callus (Crouch et al. 1993). This paper deals with the effect of growth regulators, and nutrients on the growth of callus and the yield of anthocyanin produced in vitro by selected callus cultures of *Oxalis linearis.*

Materials and methods

The basal medium consisted of MS salts and vitamins (Murashige & Skoog 1962) supplemented with 60 mM sucrose and solidified with 0.2% Gelrite. The pH of the medium was adjusted to 5.7 before autoclaving for 20 min at 121°C. Shoots obtained from mature *Oxalis linearis* plants were surface sterilised for 10 min in 0.2% $HgCl_2$ followed by five rinses in sterile distilled water. One cm long explants were placed on a shoot initiation medium in glass tubes (24 mm ϕ × 100 mm long) and sealed with parafilm. The shoot initiation medium consisted of the basal medium supplemented with 25 μM BA and 1 μM NAA. Red pigmented callus was isolated and transferred onto the basal medium containing 8 μM NAA and incubated in the dark to produce non-pigmented callus stock. The effect of growth regulators on callus growth and anthocyanin production was determined by subjecting dark grown callus to 0–32 μM NAA in combination with 0 and 8 μM BA as well as NAA (8 μM) containing medium with 8 μM BA, TZ, iP, kinetin or zeatin. To determine whether 2,4-D repressed anthocyanin production dark grown callus was incubated on 0.5–32 μM 2,4-D. The optimal sucrose concentration for anthocyanin production was

determined by growing dark grown callus on 60–360 mM sucrose. The experimental cultures were incubated for 14 days at 22–24°C under continuous cool-white fluorescent light at an intensity of 5000 Lux. Experiments were complete randomised designs and analysed accordingly (Steel & Torrey 1980).

Extraction of pigments

Freshly harvested callus was dried with paper towelling, weighed and frozen. The pigments were extracted according to Sullivan et al. (1972) and chromatographed with BAW 4:1:5, Bu-HCl 1:1 and 1% HCl according to Harborne & Gavassi (1969).

The absorbance of the anthocyanin was determined at a wavelength of 525 nm. The specific absorbance of the pigment was determined as absorbance units per gramme fresh mass. All extractions and absorbance measurements were done in triplicate.

Results

Chromatography and spectral properties of pigments

The main pigment in the extraction co-chromatographed with cyanidin-3-glucoside in three solvents used with Rf (× 100) values of 35, 28 and 16 for BAW, Bu-HCl and 1% HCl respectively.

The effect of growth regulators on callus growth and anthocyanin production

On the shoot initiation medium shoot cultures of *O. linearis* produced white or green compact callus with aggregates of loose red pigmented callus. The green callus was morphogenic and produced buds. When subcultured on several combinations of 0 and 8 μM BA and 0–32 μM NAA the red callus did not revert back to non-pigmented or green callus and lost its morphogenetic potential.

From the results in Table 1, it was clear that the anthocyanin containing callus could grow in the absence of cytokinins but not without auxins. Callus growth was substantially improved with the addition of 8–32 μM NAA. In contrast, the addition of 8 μM BA to nutrient media containing 2–32 μM NAA inhibited callus growth substantially.

The effect of growth regulators on pigment production was quite different from their effect on callus growth. Anthocyanin formation took place in the absence of growth regulators. A significant decrease in anthocyanin production was observed with an increase of NAA from 2–32 μM in the nutrient medium. However, anthocyanin production was substantially stimulated with the addition of 8 μM BA to the nutrient media containing 0–32 μM NAA . The highest anthocyanin production occurred at a combination of 8 μM BA and 2 μM NAA (Table 1). Since BA stimulated the production of anthocyanins the effect of other cytokinins on pigment production and callus growth was determined. In the presence of 8 μM NAA and 8 μM cytokinins the growth of callus was substantially decreased by BA and zeatin and to a lesser extent by kinetin and TZ. In contrast iP had a significantly stimulating effect on the growth of callus (Table 2). Pigment production was substantially increased by BA, TZ and zeatin. Kinetin

Table 1. The effect of BA and NAA on the growth and anthocyanin production of *Oxalis linearis* callus.

Growth regulators (μM)		Growth index	Specific absorbance
BA	NAA		
0	0	0.1	1.71
0	2	1.2	1.15^{a}
0	8	6.0^{b*}	0.64^{a}
0	32	6.4^{b}	0.42^{a}
8	0	0.5	2.47^{b}
8	2	0.7	2.86^{c}
8	8	4.1^{a}	2.26^{b}
8	32	3.9^{a}	2.13^{b}

* Treatments with same symbols are significantly different at a 95% level of confidence.

Table 2. The effect of cytokinins on the growth and anthocyanin production of *Oxalis linearis* callus cultured in basal medium supplemented with 8 μM NAA.

Cytokinin (8μM)	Growth index	Specific absorbance
Control	5.9	1.24
BA	2.6^{b*}	2.19^{a}
Kinetin	4.6	1.65
iP	7.7^{a}	1.13
TZ	3.6	2.10^{a}
Zeatin	1.9^{b}	2.95^{a}

* Treatments with same symbols are significantly different at a 95% level of confidence.

Table 3. The effect of 2,4-D on the growth and anthocyanin production of *Oxalis linearis* callus.

2,4-D (μM)	Growth index	Specific absorbance
0.5	3.7	1.07
2	4.8	2.19[a]
8	6.4[a]	1.16
32	6.8[a]	0.76

*Treatments with same symbols are significantly different at a 95% level of confidence.

Table 4. The effect of sucrose on the growth and anthocyanin production of *Oxalis linearis* callus.

Sucrose (mM)	Growth index	Specific absorbance
60	4.4	0.82
120	3.5	1.36[a]*
180	3.3	1.56[a]
240	3.0	1.78[a]
300	2.0[a]	3.03[b]
360	1.5[a]	3.28[b]

*Treatments with same symbols are significantly different at a 95% level of confidence.

and iP had little effect on the production of pigment (Table 2).

Callus growth was better at a concentration of 8–32 μM 2,4-D than at lower concentrations of 0.5–2 μM 2,4-D. Anthocyanin production was increased by 2,4-D to reach a peak at 2 μM 2,4-D and then decreased at higher concentrations of 8 μM and 32 μM 2,4-D (Table 3).

The effect of sucrose on callus growth and anthocyanin production

Callus growth was the best with 60 mM sucrose in the nutrient medium. At higher levels of sucrose a substantial decline in callus growth was observed with a significant decrease in growth at 300–360 mM sucrose. Pigment production was low at 60 mM sucrose. It increased up to 240 mM sucrose and was substantially higher at 300–360 mM than at lower sucrose concentrations (Table 4).

Discussion

The production of red pigmented and green compact callus as was observed with *O. linearis* was also reported for *O. erosa* (Ochatt & de Azkue 1984). As with *O. linearis* red pigmented callus of *O. erosa* was not morphogenetic in the presence of a cytokinin and auxin. The addition of gibberellic acid to the nutrient medium seemed to be essential for the induction of morphogenesis in red pigmented *Oxalis* callus (Ochatt & de Azkue 1984).

The promotion of anthocyanin production by cytokinins as was observed with *O. linearis* was also reported for *Haplopappus gracilis* (Constable et al. 1971) and *Daucus carota* (Ozeki & Komamine 1986).

The repression of anthocyanin production in *O. linearis* callus cultures by auxins such as NAA and 2,4-D confirms the reports on *Haplopappus gracilis* (Constable et al. 1971), *Daucus carota* L. cv Korudagosun (Ozeki & Komamine 1986), *Helianthus tuberosus* (Ibrahim et al. 1972) and *Petunia hybrida* cv. Violet 30 (Haagendoorn et al. 1991). Decreased concentrations of auxins that result in the increase in pigment formation was suggested to be in agreement with the hypothesis that auxins and elicitors act on the level of the plasma membrane via a decrease in the plasma membrane proton gradient (Haagendoorn et al. 1991).

The effect of elevated levels of sucrose on the inhibition of callus growth and stimulation of pigment production observed with *O. linearis* was also reported for in vitro cultures of *Daucus carota* cv. Kurodagosun (Ozeki & Komamine 1985) and *Phytolacca americana* (Sakuta et al. 1987). The repression of growth and stimulation of pigment formation by increasing sucrose concentrations was suggested to be a result of the sucrose per se as well as the change of the water potential in the sucrose solutions (Sakuta et al. 1987). Although members of the Oxalidaceae are not being used as a source of colorants in Southern Africa, this study has shown that through the use of biotechnology plants not used before could be utilised as a source of secondary products such as pigments.

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