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Induction of anthocyanin pigment in callus cultures of *Solanum melongena* L. in response to plant growth regulators and light

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Abstract
Anthocyanins are natural pigments which have various health benefits and are potential candidate for use in the pharmaceutical industries. The fruits and vegetables containing anthocyanins have proved to be beneficial for humans due to their antioxidant activity. For the present study purple brinjal (*Solanum melongena* L.) is selected as the anthocyanin source. The type of anthocyanins in the peels of this vegetable has been determined as nasunin which is identified as delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside. The present study attempts to produce pigmented callus from *in vitro* grown seedlings of purple brinjal as explants. Various combinations of phytohormones were added to the culture media and the induction of pigmented callus was studied. The effect of light intensity was also considered. The best results were recorded when Naphthalene Acetic Acid (NAA) at 1 mg L^{-1} was used with Kinetin at 0.25 and 0.1 mg L^{-1} , at a light intensity of 4000 lux. The total anthocyanin accumulation in the pigmented callus was calculated as 70 µg g^{-1}. This type of study in *S. melongena* is being reported for the first time to the best of our knowledge.

Keywords: Anthocyanins, antioxidant, phytohormones, pigmented callus, purple brinjal, *Solanum melongena.*

1. Introduction

Anthocyanins are natural pigments which give some fruits and flowers their pink, red, magenta, purple and dark blue colours. These pigments are synthesized in the cytosol and are localized in the vacuoles of the plant cells [1]. Anthocyanins have antioxidant ability and have been shown to have many health benefits [2-5]. It also protects the plants from damage caused by UV radiation. A growing number of scientific and epidemiological reports suggest that anthocyanins or the anthocyanin extracts exhibit a wide range of protective effects with potential benefits for human and animal health [6, 7]. Due to these medicinal properties, anthocyanin extracts can be used as food colorants as well as components of pharmaceutical preparations and functional foods.

But there are limitations in the supply of anthocyanin. The plant sources are very limited, long cultivation times, seasonal effects, climate variation, pest or disease attack and decreasing availability of low-cost arable land. One alternative source for the production of anthocyanins is through the use of plant cell cultures [8, 9]. Anthocyanins have been obtained from *in vitro* cultures of *Daucus carota* [10], *Ipomaea batatas* [11], *Fragaria ananassa* [12], *V. vinifera* [13], *Ajuga reptans* [14], *Catharanthus roseus* [15], *Melastoma malabathricum* [16] and others. For the present study purple brinjal has been selected as the source of anthocyanins.

Eggplant or Brinjal, is a very low calorie vegetable and has healthy nutrition profile. Botanically, it belongs to solanaceae family and named as *Solanum melongena* L. The peel or skin of the purple brinjal has significant amounts of anthocyanins. The major type of anthocyanins in purple brinjal is nasunin which is identified as delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside [17, 18] and the isolated extracts have shown to have a high antioxidant activity [19]. The plant has been successfully regenerated by *in vitro* culture methods using root [20] and leaf [21] explants and also through somatic embryogenesis [22]. But there have been no attempt to produce pigmented callus from purple brinjal. This paper reports development of pigmented callus production system from this medicinally important plant.

2. Materials and Methods

Purple brinjal seeds were purchased from Sutton and Sons (India) Pvt. Ltd, Kolkata. Different explants like stem, node and leaves from *in vitro* grown seedlings were used to initiate callus cultures.

2.1. Media preparation

MS (Murashige and Skoog) basal media were used to germinate seeds. For the initial establishment of the callus culture MS media supplemented with various combinations of auxins and cytokinins as mentioned in Table 1 were prepared. The culture media were supplemented with 30 g L^{-1} sucrose. The pH of the culture media were adjusted to 5.8 and solidified with 70 g L^{-1}

agar before autoclaving at 121^{o}C for 20 minutes. Twenty milliliters of the medium were poured into glass test tubes and autoclaved. Slants were prepared after autoclaving the medium.

2.2. Surface sterilization of seeds
Seeds of the brinjal plants were first thoroughly washed with 5% detergent solution of Tween 20 for 15 min and rinsed with double distilled water. The seeds were treated with 0.2% solution of the fungicide Bavistin (BASF India Limited) for 10 min and washed thoroughly with double distilled water. Finally the seeds were treated with 0.1% mercuric chloride solution for 30 seconds and washed thoroughly with sterile double distilled water.

2.3. Inoculation and incubation
The surface sterilized seeds were aseptically grown on sterilized media and incubated at a temperature of 22±2^{o}C and at a 16h photoperiod (provided from cool-white fluorescent tube).

2.4. Callus induction
For the initial establishment of callus culture stem, node and leaves were used as the source of explants. The explants prepared from the *in vitro* grown 3-4 weeks old seedlings were cultured for callus induction. Sterile scalpel blade (number 24) was used and the explants were excised from the seedlings and collected on a sterile petri dish and were cut into segments of 5 mm. The explants were then placed on the MS media supplemented with various concentrations and combinations of auxins and cytokinins (Table 1). The explants were cultured in test tubes and maintained in the culture room at of 22± 5^{o}C and at a 16h photoperiod. Each test tube contained single explant.

2.5. Subculturing
Observations were made till four weeks of inoculation. After successful callus initiation 5-mm^3 calli was taken from four week old callus and was placed on 20 ml MS media with the same combination of phytohormones. For each media there were five replicates. Observations were recorded every alternate day.

2.6. Anthocyanin induction
The light intensity plays an important role in anthocyanin production. In this study different intensities of fluorescent light ranging from 1000 to 4000 lux were assessed to check the effect of light intensity on pigment development.

2.7. Determination of Anthocyanin content in Fresh Callus
Fresh calli (0.5 gm) was weighed in 15 ml plastic centrifuge tube and broken to small pieces using forceps. Five milliliters of methanol containing 1% concentrated HCl at 4°C was added to the sample. The tubes were vortexed and the samples were centrifuged at 15000 g for 20 min at 4°C. Absorbance of the clear supernatant was measured at 528 nm. Anthocyanin content was calculated according to the method described by Mori *et al*, 1993 [23]. The major anthocyanin in purple brinjal has been previously identified as delphinidin. Total anthocyanin yield was expressed as (µg g^{-1}) fresh weight of callus.

3. Results
Different combinations and concentrations of plant growth hormones gave different results in the selected plant materials. The effect of various combinations and concentrations of different phytohormones on callus induction are presented in Table 1.

The calli first developed within 10-12 days of inoculation. The light intensity did not show any remarkable effect in the calli induction from explants. The nature of the callus was friable and white to light green in colour. The appearance of purple pigmented callus was observed after the third subculturing (Fig. 1a). It was also observed that cream to purple callus started appearing when the light intensity was increased from 2000 lux to 4000 lux. Light intensity of less than 2000 lux did not produce intense pigmentation.

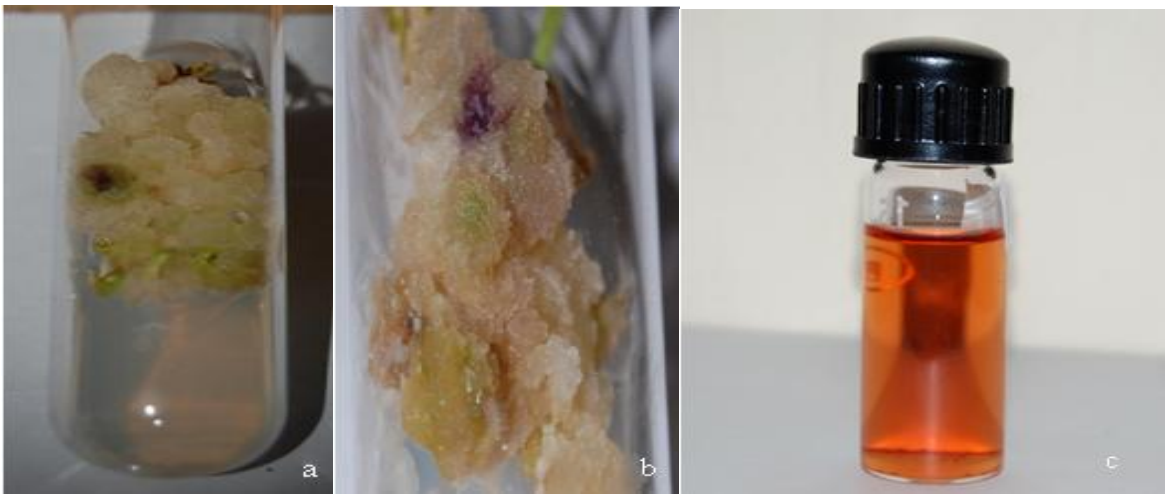

Fig. 1. Anthocyanin pigmented callus of purple brinjal, (a) Initiation of pigmented callus (b) purple pigmented callus, (c) pigment extracted from the callus.

Table 1. Effect of different plant growth hormones on callus induction

Sl. No.	Phytohormone combination	Concentration (mg L^{-1})	Days to callus initiation	Observations
1.	2,4-D+BAP	1+0.5	-	NC
		1+0.25	12	NPC
		1+0.1	10	NPC
		0.5+0.5	-	NC
		0.5+0.25	10	NPC
		0.5+0.1	10	NPC
2.	2,4-D+Kin	1+0.5	10	NPC
		1+0.25	10	NPC
		1+0.1	-	NC
		0.5+0.5	-	NC
		0.5+0.25	-	NC
		0.5+0.1	10	NPC
3.	NAA+BAP	1+0.5	-	NC
		1+0.25	10	NPC
		1+0.1	10	NPC
		0.5+0.5	10	NPC
		0.5+0.25	-	NC
		0.5+0.1	12	NPC
4.	NAA+Kin	1+0.5	12	NPC
		1+0.25	10	PC
		1+0.1	12	PC
		0.5+0.5	10	NPC
		0.5+0.25	10	NPC
		0.5+0.1	-	NC

Abbreviations used in the table:
NAA: Naphthalene Acetic Acid, 2,4-D: 2,4-Dichlorophenoxyacetic acid, Kin: Kinetin, BAP: Benzylaminopurine
NPC: Non-pigmented callus, PC: Pigmented callus, NC: No callus

Regarding production of pigmented calli, best results were recorded when NAA at 1 mg L^{-1} was used with Kinetin at 0.25 and 0.1 mg L^{-1}. Production of pigmented callus was induced after 8 weeks of initial callus induction (Fig. 1b). The total anthocyanins accumulation in the pigmented callus extract (Fig. 1c) was calculated as 70 µg g^{-1} and the absorbance spectra showed a λ max at 536.54 nm (Fig. 2). In an earlier study the peel anthocyanin extracts from the *in vivo* purple brinjals had a total anthocyanins content of 138 µg g^{-1} [19].

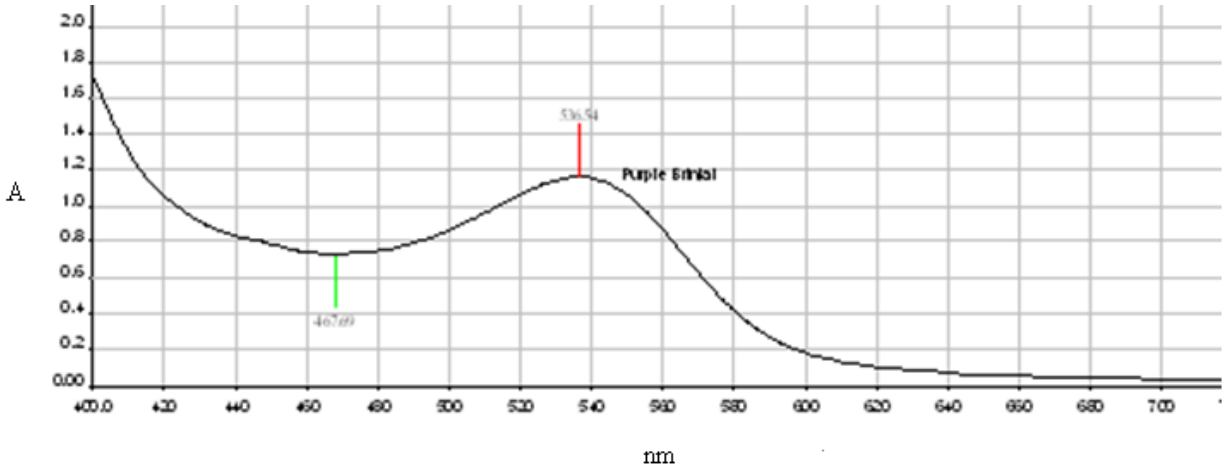

Fig. 2. Absorbance spectra of anthocyanin extracts from callus culture of purple brinjal.

4. Conclusion

Much effort has been expended in developing alternative methods to improve anthocyanin biosynthesis under *in vitro* conditions. The present study reports a suitable and reliable approach for pigment production in callus cultures of purple brinjal. The type and ratio of phytohormones had profound role as stimuli in anthocyanins accumulation. The optimized protocol which accumulated anthocyanin in the calli under investigation was developed on the general trend of cytokinin auxin combination. The light intensity also played an important role in accumulation of anthocyanins *in vivo* and *in vitro*. All these important aspects were studied in the present experiment. Based on our experimental data we conclude that NAA and Kinetin are the best phytohormone combination for the production of anthocyanin pigmented callus in purple brinjal. There is no report of such study in this plant till date to the best of our knowledge

This *in vitro* grown pigmented callus can be further used to initiate suspension cultures and various elicitors can be used to extrude the pigment in the culture medium. Since plant tissue culture proves to be an efficient method to manipulate secondary metabolite production *in vitro* this method can be used to overproduce anthocyanin pigments content than the *in vivo* anthocyanin content in the purple brinjal peels. Brinjals have many health benefits and the anthocyanin in the peels of this vegetable has shown to have antioxidant activity, hence these *in vitro* produced pigments have a potential use not only as a colourant in the pharmaceutical industries but also to control various diseases in a natural way.

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