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Botryococcus braunii, a New Elicitor for Secondary Metabolite Production in *Capsicum frutescens*

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ABSTRACT

Botryococcus braunii is a green colonial microalga which is mainly used for the production of hydrocarbons, exopolysaccharides and carotenoids. The present study revealed that some of the bioactive principles that are present in the algal extracts of *B. braunii* influence growth parameters, pigment and secondary metabolites production in *Capsicum frutescens* at different levels (1, 2, 4 and 8 mg/l). Upon treatment with different *B. braunii* extracts seed germination, root length, shoot length and leaf length were enhanced in both light and dark conditions. A two-fold increase in germination occurred when 8 mg/l of *B. braunii* extract was applied, both in the light and in the dark. Similarly, a 1-1.5-fold increased in shoot and leaf length was observed with 8 mg/l of *B. braunii* extract, a 2-3-fold increase in chlorophyll and a 2-fold increase in carotenoid content after exposure of *C. frutescens* to 8 mg/l of *B. braunii* extract. Vanillylamine was enhanced by 1-1.5-fold, capsaicin by 2-2.5-fold and 2-fold compared to the control after 15 days of incubation with the maximum concentration of *B. braunii*.

Keywords: *B. braunii*, *C. frutescens*, capsaicin, microalga, vanillylamine

INTRODUCTION

Botryococcus braunii is a green colonial microalga belonging to the family Chlorophyceae and is grouped into three different races A, B and L depending on the type of hydrocarbons they synthesize (Metzger and Largeau 2005). This alga is mainly known for the production of hydrocarbons, exopolysaccharides and carotenoids. *B. braunii* undergoes a color change because of the accumulation of secondary carotenoids in the matrix. The presence of carotenoids is more pronounced in races B and L (Grung *et al*. 1989). In the linear stage of growth, both races produce almost equal amounts of β-carotene, echinenone, canthaxanthin, lutein, violaxanthin, loroxanthin, and neoxanthin. However, lutein is the major carotenoid (22 and 29%) reported in the linear phase of these races. Some newly identified carotenoids such as botryoxanthin-A (Okada *et al*. 1996), botryoxanthin-B, and α-botryoxanthin (Okada *et al*. 1998), braunixanthin 1 and 2 (Okada *et al*. 1997) isolated from race B may contribute to the color of algal colonies. *Botryococcus* extracts have also been evaluated for elicitation of secondary metabolites and have also been reported to show growth-promoting effects on roots of *Tagetes erecta*at the 8 ppm level (Murakami 2000). Phycocyanin, a constituent pigment of cyanophycean algae, has been reported to be an elicitor of secondary metabolites (Ramachandra Rao *et al*. 1996). *Capsicum* sp. produces the pungent principle capsaicin which has immense use as a food additive and pharmaceutically importance compound. So far, researchers have reported capsaicin as an antioxidant, antiarthritic, and gastroprotective agent, anticancer agent and also as an analgesic (Prasad *et al*. 2005). Cell cultures and immobilized cell cultures of *Capsicum* have shown the potential for continuous production of capsaicin in column reactors and in which they also have the ability to biotransform phenylpropanoids into capsaicin (Johnson *et al*. 1992). Capsaicin synthase is a terminal enzyme of the capsaicin biosynthetic pathway that catalyses the condensation reaction between vanillylamine and nonanoic acid to form capsaicin (Prasad *et al*. 2005). The present study aimed to evaluate the effect of green alga *B. braunii* extracts on the growth and secondary metabolite production in *Capsicum frutescens* callus culture.

MATERIALS AND METHODS

Chemicals

All of the chemicals and solvents were of analytical and high-performance liquid chromatography (HPLC) grade and were obtained from Ranbaxy Fine Chemicals Ltd. (Mumbai, India). Standard astaxanthin, lutein, vanillylamine, vanillin, capsaicin and β-carotene were obtained from Sigma Chemicals Co. (St. Louis, MO).

Algal culture

B. braunii (LB-572) was obtained from the University of Texas, U.S.A. Stock cultures were maintained on both liquid and agar slants of modified Chu13 medium (Largeau *et al*. 1980) by subculturing at two-week intervals. The cultures were incubated at 25 ± 2 °C under 1.2 ± 0.2 Klux light intensity and a 16 h photoperiod.

Media and culture conditions

A set of 250 ml Erlenmeyer conical flasks were taken and 80 ml of modified Chu13 medium was distributed and two-weeks old culture of *B. braunii* (LB-572) was used as inoculum at 20% (v/v). The culture flasks were incubated at 25 ± 2°C under 1.2 ± 0.2 klux light intensity and a 16-h photoperiod. The cultures were harvested by centrifugation at 5,000 × *g* and the cells were washed with distilled water and the pellet was freeze-dried.

Received: 8 March, 2010. Accepted: 8 August, 2010.

Original Research Paper

Estimation of carotenoids and total chlorophylls

Carotenoids and chlorophylls (Chl) were extracted from *B. braunii* according to the procedure of Lichtenthaler *et al*. (1987). *B. braunii* was ground with ice-cold acetone using a mortar and pestle and carotenoids and Chls were extracted with acetone repeatedly until the extract became colorless. The pooled extract absorbance was read (Shimadzu 160A) at 450, 645 and 661.5 nm. The acetone extract was concentrated by flash evaporation (Buchi Rotavapor R205, Germany) and further dried under nitrogen gas. The residue was redissolved in dimethylsulfoxide (DMSO) and taken for different concentration of elicitor studies.

Hydrocarbon extraction

The dry biomass was homogenized in a mortar and pestle with *n*-hexane for 15 min and centrifuged. The extraction process was repeated twice and supernatant was transformed to a preweighed glass vial and evaporated under a stream of nitrogen to complete dryness. The quantity of residue was measured gravimetrically and expressed as dry weight percentage (Dayananda *et al*. 2005).

Hydrocarbon analysis by gas chromatography

Hydrocarbon extract was purified by column chromatography on a silica gel. The hydrocarbon sample was analyzed using an ELITE-5 capillary column. The conditions used were oven temperature initially at 130°C for 5 min and then increased to 200°C at a rate of 8°C/min and maintained for 2 min and then increased up to 280°C at a rate of 5°C/min and maintained for 15 min. The injector port and the detector temperatures were 240 and 250°C, respectively (Dayananda *et al*. 2005).

HPLC analysis of carotenoids in *B. braunii* extract

The carotenoid profiles of *B. braunii* extract were analysed by HPLC using a reversed phase C18 column (3.9 × 300 mm, µBond Pack, Waters, Ireland) with an isocratic solvent system consisting of acetonitrile: methanol: dichloromethane (60: 20: 20) at a flow rate of 1.0 ml/min and detected at 450 nm according to the method of Ranga Rao *et al*. (2006).

Determination of total phenolic compounds

The concentration of total phenolic compounds in the extracts was determined according to the method of Taga *et al*. (1984) and expressed as caffeic acid equivalents. In brief, samples and standards were prepared in acidified (3 g/l HCl) methanol/water 60: 40 (v/v) and 100 µl of each were added separately to 2 ml of 2% sodium carbonate. After 5 min, 100 µl of 50% Folin–Ciocalteu reagent was added and the mixture was allowed to stand at room temperature for 30 min. Absorbance was measured at 750 nm using a spectrophotometer (Shimadzu 160A). The blank consisted of all reagents and solvents without sample or standard. Standard caffeic acid was prepared at concentrations of 10-100 µg/ml. The phenolic concentration in the alga extract was determined by comparison with the standards.

Callus induction and maintenance

Callus cultures were initiated from the leaves and hypocotyls of 25-30 days old seedlings of *C. frutescens* Var. KT-OC (DRDO Pithoragarh) on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 2 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 mg/l Kinetin and 3% (w/v) sucrose. The cultures were incubated at 25 ± 2°C under continuous light at 1.2 ± 0.2 klux (Johnson *et al*. 1992). The callus thus obtained was maintained in the same medium by regular sub-culturing at 3-week intervals and 3-week old callus was used for the experiment because of maximum concentration of secondary metabolites.

Elicitor preparation

Lyophilized algal biomass (2 g) was extracted with acetone (100 ml) in a mortar and pestle and the process was repeated until the biomass turns colorless. Extracts were pooled and concentrated in a nitrogen stream and redissolved in DMSO. Aliquots of filter-sterilized *B. braunii* extracts (1-8 mg/l) were added to the culture tubes (25 × 125 mm) with filter paper bridges (Whatman No.1) containing MS medium whereas the control tubes received the same volumes of DMSO. Fresh callus (500 ± 30 mg) was transferred to each tube and incubated at 25 ± 2°C under continuous light at 1.2 ± 0.2 klux. Callus cultures were harvested at 5-day intervals to study the effect of *B. braunii* extracts on callus growth and capsaicinoids accumulation.

Growth measurement

Growth of the callus was measured in terms of fresh and dry weight as reported by Ramachandra Rao *et al*. (1996).

Extraction of capsaicinoids and other secondary metabolites

Capsaicinoids were extracted from callus tissue (known quantity) was macerated in a mortar with 5 mL of ethyl acetate in the presence of neutralized glass powder and filtered on Advantec (Dublin, CA) no. 2 filter paper. The residue was then rinsed three more times with 5 ml of ethyl acetate. The supernatant was collected and brought to a volume of 25 ml with methanol. The filtrate was centrifuged at 18,000 × *g* for 10 min at 1°C; the extract was dried in a nitrogen stream. All capsaicinoid extracts were filtered through a 0.45-µm Millipore nylon filter (Bedford, MA) before HPLC analysis.

Analysis of capsaicinoids by HPLC

Capsaicinoid content was analysed by HPLC with a C_{18} column (Shimadzu LC-10A, Japan). The mobile phase consisted of acetonitrile: water (70: 30) and 1% acetic acid at a flow rate of 1 ml/min and the sample was detected at 280 nm. The capsaicinoids in the samples were determined by comparison with the retention times of authentic standards such as vanillylamine, vanillin and capsaicin (Johnson *et al*. 1992).

Statistical analysis

All experiments were performed in triplicate and the data presented are the average of three independent experiments with standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corp., Redmond, WA), and mean separations were performed by Duncan's Multiple-Range Test (DMRT) at $P < 0.05$.

RESULTS

Carotenoids, chlorophylls, and phenolics in *B. braunii* extract

B. braunii extract was analysed for total Chls, carotenoids and phenolics. Chls (15.78-20.54 µg/mg), carotenoids (6.7 to 8.4 µg/mg) and phenolics (145 ± 1.24 µg/mg) were estimated in the *B. braunii* extract.

HPLC profile of *B. braunii* extract

The carotenoid profile of *B. braunii* culture grown in Chu13 media were analysed by HPLC, as shown in **Fig. 1**. The order of carotenoids eluted under isocratic conditions through a C_{18} column were first xanthophylls then carotenoids. Lutein, astaxanthin and β-carotene were identified using authentic standards. Xanthophylls and carotenoids present in *B. braunii* extract were lutein (75%), astaxanthin (5%), and β-carotene (1.96%).

Hydrocarbon content in *B. braunii* extract

Hydrocarbon content in *B. braunii* was 25-28%. Hydrocarbons (C_{20-24}) content was 36.91 ± 0.36%. The hydrocarbon profile, as analyzed by GC, indicated only a marginal influ-

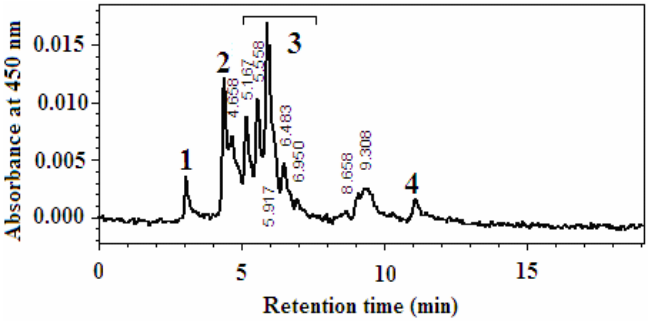

Fig. 1 HPLC profile of *B. braunii*. (1) Astaxathin, (2) Lutein, (3) Unidentified peaks and (4) β-carotene.

Table 1 Hydrocarbon content in *B. braunii* (LB-572).

B. braunii	Less than C_{20} (%)	Higher than C_{20-24} (%)	Less than C_{30} (%)	Higher than C_{30} (%)
LB-572	23.20 ± 0.14 b	36.91 ± 0.36 a	5.11 ± 0.33 c	33.51 ± 0.12 a

Values are expressed as mean ± SD (n=3). Values are not sharing a similar superscript within the same row are significantly different ($P < 0.05$) as determined by DMRT.

ence on the relative proportion of hydrocarbons lower than C_{30} and higher than C_{30} as shown in **Table 1**.

Effect of *B. braunii* extract on growth parameters of *C. frutescens*

There was a two-fold increase in seed germination in both light and dark conditions at 8 mg/l of *B. braunii* extract and a one-fold increase in root length at 8 mg/l, compared with the control. Similarly, shoot and leaf length increased 1-1.5-fold in both light and dark conditions compared with the control. *C. frutescens* increased with increasing concentration of *B. braunii* extracts (**Table 2**).

Effect of *B. braunii* extract on chlorophyll and pigment production in *C. frutescens*

Both Chl and carotenoid content was observed in *C. frutescens* when treated with *B. braunii* extract. The total Chl content increased 2-3 fold after 15 days *C. frutescens* culture when 8 mg/l *B. braunii* extract was used, compared with control samples (**Table 3**). The carotenoid content increased with increasing concentration of *B. braunii* extract levels and maximum carotenoid content (0.18 mg/g) was observed with 8 mg/l *B. braunii* extract (**Table 4**).

Effect of *B. braunii* extract on metabolite production in *C. frutescens*

The extract obtained from *B. braunii* was used as an elicitor in *Capsicum* for the production of major metabolites viz. capsaicin, vanillin, and vanillylamine. Vanillin and vanillylamine, which are intermediates in the capsaicin biosynthesis pathway, increased during the experimental period and this was reflected in the enhancement of capsaicin in the extract-treated callus cultures (**Table 5**). Vanillin, vanillyl-

Table 3 Effect of *B. braunii* on chlorophyll content in *C. frutescens*.

	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)
5 days			
Control	0.39 ± 0.02 d	0.32 ± 0.08 d	0.70 ± 0.08 d
2 ppm	0.40 ± 0.05 d	0.38 ± 0.03 d	0.71 ± 0.06 d
4 ppm	0.73 ± 0.04 c	0.40 ± 0.09 d	1.13 ± 0.10 c
8 ppm	1.17 ± 0.19 b	0.69 ± 0.07 b	1.85 ± 0.21 b
10 days			
Control	0.49 ± 0.08 d	0.43 ± 0.03 d	0.91 ± 0.07 d
2 ppm	0.48 ± 0.03 d	0.42 ± 0.06 d	0.93 ± 0.06 d
4 ppm	0.83 ± 0.06 c	0.50 ± 0.07 c	1.33 ± 0.17 c
8 ppm	1.24 ± 0.16 b	0.86 ± 0.04 a	2.12 ± 0.15 a
15 days			
Control	0.59 ± 0.09 d	0.38 ± 0.05 d	0.98 ± 0.09 d
2 ppm	0.74 ± 0.07 c	0.49 ± 0.08 c	1.23 ± 0.16 c
4 ppm	1.23 ± 0.13 b	0.67 ± 0.09 b	1.92 ± 0.13 b
8 ppm	1.45 ± 0.18 a	0.99 ± 0.08 a	2.43 ± 0.05 a

Values are expressed as mean ± SD (n=3). Values are not sharing a similar superscript within the same column are significantly different ($P < 0.05$) as determined by DMRT.

Table 4 Effect of *B. braunii* on carotenoid production in *C. frutescens*

	Carotenoid content (mg/g)		
	5 days	10 days	15 days
Control	0.038 ± 0.008 d	0.058 ± 0.006 d	0.120 ± 0.041 d
2 ppm	0.045 ± 0.007 c	0.087 ± 0.009 c	0.134 ± 0.027 c
4 ppm	0.098 ± 0.002 b	0.126 ± 0.04 b	0.156 ± 0.031 b
8ppm	0.123 ± 0.014 a	0.152 ± 0.06 a	0.187 ± 0.028 a

Values are expressed as mean ± SD (n=3). Values are not sharing a similar superscript within the same column are significantly different ($P < 0.05$) as determined by DMRT.

Table 5 Elicitation of *B. braunii* on metabolite production in *C. frutescens*.

	Vanillin (µg/g)	Vanillylamine (µg/g)	Capsaicin (µg/g)
5 Days			
Control	0.48 ± 0.03 d	1.28 ± 0.36 d	1.56 ± 0.23 d
1 ppm	0.51 ± 0.08 d	1.89 ± 0.08 d	2.45 ± 0.19 d
2 ppm	0.98 ± 0.05 b	2.34 ± 0.21 c	5.02 ± 0.24 c
4 ppm	1.04 ± 0.16 b	3.65 ± 0.33 b	6.13 ± 0.33 b
8 ppm	1.26 ± 0.17 a	5.89 ± 0.45 a	7.21 ± 0.28 a
10 Days			
Control	0.95 ± 0.10 c	1.19 ± 0.13d	5.21 ± 0.19 c
1 ppm	1.26 ± 0.12 b	2.56 ± 0.21 c	4.63 ± 0.21 c
2 ppm	1.59 ± 0.15 b	3.12 ± 0.17 b	8.26 ± 0.25 b
4 ppm	2.63 ± 0.23 a	4.25 ± 0.92 b	9.53 ± 0.30 b
8 ppm	2.74 ± 0.37 a	8.96 ± 1.52 a	12.40 ± 0.20 a
15 Days			
Control	1.18 ± 0.27 d	2.31 ± 0.15 d	9.48 ± 1.24 d
1 ppm	1.63 ± 0.34 c	4.87 ± 0.28 c	13.26 ± 1.19 c
2 ppm	2.13 ± 0.29 b	5.68 ± 0.32 c	14.68 ± 2.08 c
4 ppm	2.54 ± 0.43 b	7.98 ± 1.09 b	18.51 ± 2.13 b
8 ppm	3.46 ± 0.21 a	12.35 ± 2.31 a	20.91 ± 0.18 a

Values are expressed as mean ± SD (n=3). Values are not sharing a similar superscript within the same column are significantly different ($P < 0.05$) as determined by DMRT.

Table 2 Effect of *B. braunii* on growth parameters of *C. frutescens*.

	Light				Dark			
	Control	2 (mg/l)	4 (mg/l)	8 (mg/l)	Control	2 (mg/l)	4 (mg/l)	8 (mg/l)
No of seed inoculated	25	25	25	25	25	25	25	25
Seeds germinated (no)	8.5 ± 1.0 a	2.1 ± 0.31 b	4.21 ± 0.34 b	15.01 ± 1.23 a	7.01 ± 1.24 b	8.04 ± 1.10 a	10.12 ± 1.02 a	14.09 ± 2.13 a
Root length (cm)	4.2 ± 0.52 b	5.32 ± 0.22 a	5.2 ± 0.65 a	5.9 ± 0.10 b	6.91 ± 0.26 b	4.3 ± 0.15 b	5.15 ± 0.56b	5.83 ± 0.92 b
Shoot length (cm)	3.7 ± 0.61 c	4.50 ± 0.28 a	4.9 ± 0.23 b	6.5 ± 0.38 b	13.12 ± 0.41 a	4.7 ± 0.38 b	4.80 ± 0.31 c	5.54 ± 0.43 b
Leaf length (cm)	2.1 ± 0.23 d	2.55 ± 0.08 ab	2.7 ± 0.14 c	3.2 ± 0.34 bc	2.08 ± 0.09 c	1.5 ± 0.27 c	1.65 ± 0.28 a	2.91 ± 0.55 c
Leaf style	2.2 ± 0.31 d	2.16 ± 0.14 b	2.3 ± 0.16 c	2.1 ± 0.18 c	2.03 ± 0.13 c	2.12 ± 0.25 bc	2.13 ± 0.42 d	2.56 ± 0.30 c

Values are expressed as mean ± SD (n=3). Values are not sharing a similar superscript within the same column are significantly different ($P < 0.05$) as determined by DMRT test.

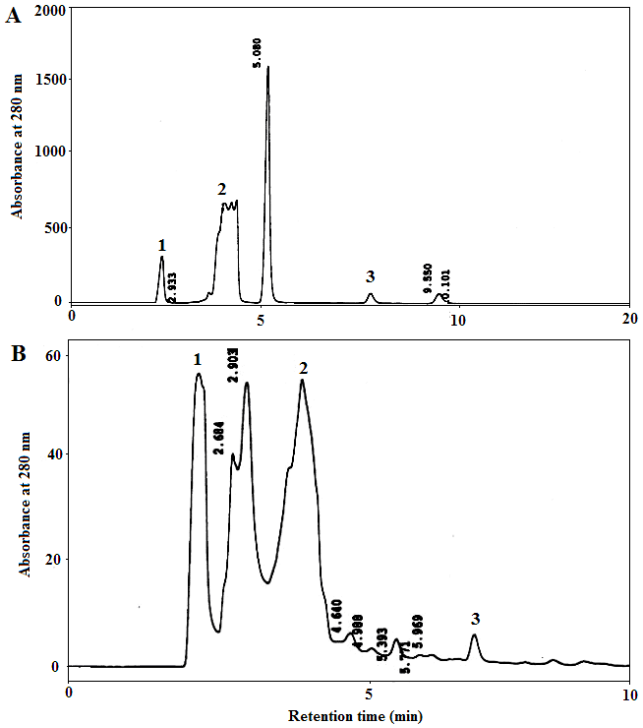

Fig. 2 HPLC profile of (**A**) standards and (**B**) treated callus cultures of *C. frutescens*. (1) Vanillin, (2) vanillylamine and (3) capsaicin.

amine and capsaicin were identified and quantified by HPLC as shown in **Fig. 2A-B**. The results indicate that *B. braunii* extracts had some bioactive principles responsible for increasing secondary metabolite production in plant tissue cultures. Vanillylamine was a remarkable elicitor when 8 mg/l of extracts of *B. braunii* were used with respect to the control with 15 days of culture incubation.

Capsaicin content increased in the *Capsicum* suspension cultures as *B. braunii* extract concentration increase from 1 to 8 mg/l and was maximum of 2.5-fold with respect to the control at 8 mg/l. Similarly, the vanillin content at 8 mg/l extract in capsicum callus was almost double that of the control after 14 days of incubation.

Analysis of capsaicinoids by HPLC

Vanillylamine, vanillin and capsaicin were observed in *C. frutescens* at different levels (1-8 mg/l) of *B. braunii* extracts when metabolites were analyzed by HPLC. Increased Vanillylamine increased 1.5-fold, capsaicin 2.5-fold and vanillin 2-fold more than the control after 15 days of incubation with the maximum concentration of *B. braunii.*

DISCUSSION

Enhancement of secondary metabolites by elicitation is one of the few strategies which is gaining commercial application. Elicitors are chemical compounds, either biotic or abiotic, which on contact with higher plants trigger the production of pigments, flavones, phytoalexins and other defense related secondary metabolites (Savitha *et al*. 2006). Elicitors are signals triggering the formation of secondary metabolites. Elicitors are believed to act by ionic communication, which can be brought about by changes in pH, electrolyte leakage, depolarization or inhibition of electrogenic ion pump (Katou *et al.* 1982). The extracts of *B. braunii* are shown to have an elicitor property for secondary metabolite production in plant cell culture systems (Murakami *et al*. 2000). Mercier *et al*. (2001) also studied the elicitor effects of two algal polysaccharides, laminarin and carrageenans, on defense responses and signaling in tobacco plants. However, in general, secondary metabolites will be triggered in higher plants in response to external physical, chemical and biological stimuli (Sudha and Ravishankar 2003). Exogenous ABA enhances the resistance or the susceptibility of various plants to pathogens (Mauch-Mani and Mauch 2005; Robert-Seilaniantz *et al*. 2007). Mukundan and Hjortso (1990) reported increased thiophene accumulation by exposure of hairy roots of *Tagetes patula* to *Aspergillus niger.* Toppan *et al*. (1984) reported that fungal glycoproteins and carbohydrate elicitors might bind to receptor sites on plasma membrane and elicit a phytochemical response. Some abiotic elicitors and chitosan were shown to *Fusarium solanif.* sp. with nuclear DNA (Hadwiger *et al*. 1981). There are reports on improvement of indole alkaloid production in cell cultures of *Catharanthus roseus* treated by various elicitors (Zhao *et al*. 2005). The content of alkaloids in *C. roseus* have been found influenced by individual factor, such as stage of plant growth and triadimefon, a plant growth regulator, treatment (Jaleel *et al*. 2006) There is evidence which shows that the mechanism of elicitation can in some cases operate at the level of gene expression (Dixon 1986). Hanagata *et al*. (1994) reported that aqueous extracts of blue green algae-*Anabaena cylindrica* and *Nostoc linckia* increased (10-fold) the red pigment production in *Carthamus tinctorius* cell cultures. The algal extracts obtained from *Synechococcus* have also been used to enhance somatic embryogenesis, germination of artificial seeds and root as well as shoot development of *Daucus carota* (Wake *et al*. 1991, 1992). Fingerhut *et al*. (1984) reported the increased growth of *Rhizobium japonicum* by the extract of *Scenedesmus obliquus*. Similarly, the growth of shoots and roots was enhanced by the addition of *Synechococcus* extract in carrot somatic embryos (Wake *et al*. 1989). Luczkiewicz (2008) showed that effect of elicitors on isoflavanoids production is always beneficial and explored for many members of fabaceae. Hanagata *et al*. (1994) reported a 10-fold increase in pigment accumulation in *C. tinctorius* cultures upon the addition of *Nostoc linckia* extract. Ramachandra Rao *et al*. (1996) demonstrated that the addition of phycobiliprotein from *Spirulina platensis* increased the yields of capsaicin and anthocyanin 2-fold in *C. frutescens* and *D. carota* cultures, respectively whereas elicitation of thiophene was achieved a 1.2-fold increase by the use of aqueous extracts of *H. pluvialis*.

CONCLUDING REMARKS

The elicitor preparation included steps of autoclaving and centrifugation to separate out the denatured proteins, it would be logical to assume that a soluble component of the cell wall or cell inclusion which is no the at sensitive could be responsible for the elicitation process. However one cannot rule out the possibility of trace amounts of denatured proteins influencing enhancement of secondary metabolites. Further work is in progress to characterize the elicitor. These results indicated that *B. braunii* extracts were obtained here are highly beneficial for the production of growth, pigments, capsaicin and vanillin in plant tissue cultures of *C. frutescens*.

ACKNOWLEDGEMENTS

Authors are grateful to Department of Biotechnology, Government of India, New Delhi, for their financial support. The award of Senior Research Fellowship to AS, CD and AR by the Council of Scientific & Industrial Research (CSIR) and Indian Council of Medical Research (ICMR), New Delhi, is gratefully acknowledged.

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