Effect of cyanobacterial elicitor on neem cell suspension cultures

B. Poornasri Devi, A. Vimala, Isha Sai and S. Chandra *
Dept. of Biosciences, Sri Sathya Sai University, Anantapur Campus, Anantapur-5150 001, A.P., India
**P.G. Dept. of Botany, Queen Mary's College, Chennai 600004,T.N.*
b.psridevi@yahoo.com; laks1956@yahoo.co.in

Abstract: The present study aimed to elucidate the effect of cyanobacterial elicitor derived from *Anabaena sp.* and *Nostoc carneum* on neem cell suspensions evolved from superior callus lines of *Azadirachta indica* A. Juss elite KN-1 accession with 12.88 mg/gm azadirachtin in its seed. Neem cell suspension cultures maintained in Murashige and Skoog liquid medium were treated with 265 cells/ml and 530 cells/ml homogenate of the elicitor on the third day. Growth of neem callus in terms of biomass revealed 5 fold enhancements when treated with 530 cells/ml elicitor on tenth day in callus line-1. In callus line-2 the log phase extended till tenth day recording a 6 fold increase with 265 cells/ml elicitor concentration. The type of callus line and concentration of the elicitor influence the biomass growth of neem callus in suspension culture. Enhanced azadirachtin production was achieved by algal elicitor treatment (as confirmed by HPLC analysis) and this study makes a step forward towards industrial manufacturing of the neem alkaloid.
Keywords: *Anabaena sp., Nostoc carneum,* Neem, Azadirachtin, Elicitor, Suspension Culture.

Introduction

Secondary metabolites such as alkaloids, flavanoids, glycosides, oils, tannins, resins are isolated from wild or cultivated plants because their chemical synthesis is economically infeasible. Plant cells show physiological and morphological responses to microbial, physical or chemical factors which are known as 'elicitors'. Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plants to ensure their survival persistence and competitiveness. Elicitors are abiotic and biotic, or on their basis of 'origin' like exogenous elicitors and endogenous elicitors. Abiotic elicitors are the substances of non-biological origin, i.e. inorganic salts and physical factors acting as elicitors like Cu^{2+} and Cd^{2+} ions, Ca^{2+} and high pH, where as biotic elicitors are substances of biological origin including polysaccharides derived from plant cell walls (pectin or cellulose) and microorganisms (chitin or glucans and glycol proteins or G-proteins or intercellular proteins). Exogenous elicitors are substances originated outside the cell like polysaccharides, polyamines and fatty acids, where as endogenous elicitors are substances originated inside the cell (Namdeo, 2007).

Elicitation of cell cultures with biotic elicitor and signal compounds has been recognized as an important strategy for enhancement of secondary metabolites (Savitha *et al.,* 2006). The enhanced production of secondary metabolites from plant cell cultures through elicitation has opened up a new area of research which could have important economical benefits for pharmaceutical industry. Neem (*Azadirachta indica* A. Juss Family Meliaceae) has spread to many parts of the world in the tropical and subtropical regions where there is no frost. Various parts of the tree have been in use for several millennia for medicinal, pharmaceutical and agricultural purposes.

Azadirachtin, an important bio-pesticide is one of the most active principles obtained from the neem seeds. In order to overcome the difficulties concerning variation in its source, availability, genetic stability and maintenance of purity, *in-vitro* production (Prakash *et al.,* 2006; Sujanya *et al*., 2008) through tissue culture techniques is considered to be suitable alternative. Biotic elicitors of fungal origin revealed to be effective on growth as well as the production of azadirachtin in neem cell suspension cultures. Since there is only a scanty reports regarding algal/cyanobacterial elicitors, the present investigation on the effect of cyanobacterial elicitor on neem cell suspension cultures was carried out to understand the biochemical responses of the plant cells when challenged by the elicitor.

The present study investigated the positive role of cyanobacterial elicitors (*Anabaena sp.* and *Nostoc carneum*) on the biomass and its protein content of neem cell suspensions and the efficiency of elicitor to enhance the production of azadirachtin.

Material and Methods

Experimental material

Elite neem accessions KN1 with high azadirachtin (AZ) 12.88 mg/g, in its seed was identified after an extensive survey of Anantapur District, Andhra Pradesh, India from the provenance of Karnatakanagepalli.

Surface sterilization of explants

The *in-vivo* explants such as stems, immature embryos were washed with running water and dettol for 5 minutes and further rinsed with 5% Extran MA 02 for 5 minutes. It is followed by several washes in running tap water for 30 - 45

minutes. Further surface sterilization was carried out in the laminar airflow chamber where the explants were rinsed with 70% ethanol for 30 seconds and were subsequently sterilized with 0.1% mercuric chloride for 5 minutes for stem explants and 6 minutes for immature embryos. Finally the explants were washed twice with sterile distilled water for ten minutes per wash and used for inoculation.

Establishment of AZ-rich callus cell lines

KN1 superior callus line germplasms were established after initial screening. The cotyledons were removed carefully using a scalpel from the surface sterilized fruits and were inoculated on M.S medium (Murashige & Skoog, 1962) supplemented with 3% sucrose.

Establishment of neem callus cell suspension cultures

About 15 – 20 ml of callus cell suspension was made out of 2.5gm of brownish friable callus homogenized in 100ml liquid medium. The suspension cultures were maintained at 90 rpm on a rotary shaker at 25±2^{0}C, 3000 Lux and 16/8 hours photoperiod or total dark period.

Cyanobacterial elicitor- preparation

Pure cultures of two cyanobacteria of Nostocaceae family, namely, *Anabaena sp.* and *Nostoc carneum* were cultured in Bold's Basal medium (Bischoff & Bold, 1963). The algal/cyanobacterial density as assessed by haemocytometer count was 530/ml for *Anabaena sp.* and 1670/ml for *Nostoc carneum*. 10ml of the algal/cyanobacterial cultures were autoclaved at 15 lb pressure and 121^{0}C temperature for 30minutes and homogenised were used as elicitors.

Cyanobacterial elicitor- treatment

Two to three days old neem cell suspension cultures were treated with cyanobacterial elicitors of the following concentrations and their effect on biomass and other parameters were assessed periodically.

- Density of the elicitor *Anabaena* 265/ml and 530/ml
- Density of the elicitor *Nostoc carneum* 1670/ml

Quantification of biomass growth

Biomass change was quantified in all the variants of cell suspensions established from *in vitro* callus lines of KN1 neem accessions. The various parameters scored for assessing biomass were: fresh weight, (packed cell weight/ settled cell weight) and dry weight.

Packed cell weight: A 2ml of algal/cyanobacterial cell suspension was withdrawn and centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the packed cell weight was recorded from the weight of the pellets representing under aseptic conditions.

Growth efficiency: The efficiency of growth was assessed by average growth rate (QX):

QX = (maximum cell density) - (initial cell density)/ (Initial cell density) (culture time in weeks).

Specific Growth Rate: Specific growth rate (μ) was calculated from (lnX-lnXo) versus time (t) plot where, Xo is the initial biomass at the time of inoculation, X is the biomass concentration estimated by measuring the fresh weight on different days of culture and 't' is time period of the culture during the log phase of the culture.

$$\mu = \ln X - \ln Xo / t$$

Growth Index: It is the ratio of the net increase in biomass to the initial biomass over a given period of time $N = \frac{X_f - X_1}{X_1}$

N = Growth index (dimensionless number) X_f = Final biomass X_1 = Initial biomass

Quantitative analysis of azadirachtin using HPLC

Azadirachtin production from *in-vitro* neem cell suspension cultures was quantified by HPLC analysis. The intracellular and extracellular components were homogenized using a high speed micro tissue homogenizer. The homogenized cell-free components were centrifuged at 13,000 rpm, -4°C for 10 minutes and extracted into HPLC grade methanol. The supernatant collected was filtered through Sep-pak C-18 cartridge. A portion of the filtrate was subjected to HPLC analysis on Shimadzu LC-AT series, the mobile phase being acetonitrile: water, 35:65 at a flow rate of 1ml/min with reverse phase analytical Chromega C-18 column (50mm length X 4.6mm diameter) or Phenomenex C-18 column (250mm length X 4.6mm diameter) with azadirachtin retention time at 6.7 minutes or 19.5

Table 1. Effect of cyanobacterial elicitor (Anabaena sp.) on the biomass of cell suspension cultures of neem variety KN-1

Duration (in days)	Fresh weight (gms/l)					
	Callus Line-1			Callus Line-2		
	Control	265 cells/ml Elicitor*	530 cells/ml Elicitor*	Control	265 cells/ml Elicitor*	530 cells/ml Elicitor*
1	25.0± 0.0	25.0± 0.0	25.0± 0.0	25.0± 0.0	25.0± 0.0	25.0 ± 0.0
3	111.6±4.0	111.6±4.0	111.6±4.0	55.6 ±2.3	55.6±2.3	55.6±2.3
8	96.4±3.0	87.9±2.4	133.4±1.7	144.3±4.5	137.6±1.8	62.5±2.1
10	25.0±2.0	38.9±1.3	104.6±2.8	62.4±1.1	155.7±3.2	53.2±4.1

** Addition of elictor on 3rd day*

Table 2. Effect of cyanobacterial (Anabaena sp.) elicitor on the growth rate of cell suspension cultures of neem variety KN-1

Parameter		Average growth rate	Duration in days				
			3	8		10	
			SG	SG	GI	SG	GI
Callus line-1	Control	0.346	1.458	0.169	2.856	0.001	0
	265 cells/ml Elicitor*	0.251	0.251	0.157	2.516	0.044	0.556
	530 cells/ml Elicitor*	0.436	0.436	0.209	4.336	0.143	3.184
Callus line-2	Control	0.477	1.213	0.219	4.772	0.092	1.496
	265 cells/ml Elicitor*	0.522	0.522	0.213	4.504	0.183	5.228
	530 cells/ml Elicitor*	0.150	0.150	0.115	1.500	0.075	1.128

*SG –Specific growth rate; GI – Growth index; *Addition of elicitor on 3rd day*

minutes respectively. Azadirachtin was detected at 215nm by SPD-10AVP UV-VIS detector and quantified by comparing with the authentic (azadirachtin 95%, Sigma, USA Catalogue No.A-7430).

Results

Effect of cyanobacterial elicitor (Anabaena sp.) on the biomass and growth rate of cell suspension cultures of neem variety KN-1

A study using *Anabaena sp.* as elicitor on two cell lines in two concentrations i.e., 265cells/ml and 530cells/ml (Table1) revealed, 530cells/ml concentration as optimum for biomass growth (133.4gm/l) in callus line-1, whereas lower concentration (265/ml) of the elicitor gave optimal results in callus line-2 (155.7gm/l). Growth index as given in Table-2 reveals 5.2 fold increase over the initial weight of the biomass which is highest when compared to any other concentration or growth period. Plant cell cultures take longer time (3-4 weeks) for doubling the growth with low growth rates (Ryu & Lee, 1990). The algal/cyanobacterial elicitor is considered as an excellent biomass inducer compared to fungal elicitor (Vijayashree, 2007).

Table 3. Effect of cyanobacterial elicitor (Nostoc carneum) on the biomass of cell suspension cultures of neem variety KN-1

Duration (in days)	Fresh weight (gms/l)	
	Control	1670cells/ml elicitor*
0	25.0± 0.00	25 ± 0.00
2	48.09 ± 3.57	60 ± 5.16
3	46.0 ± 6.00	80 ± 0.00
4	45.0 ± 5.00	55 ± 5.00
5	40.0 ± 6.00	55 ± 5.00
7	40.0 ± 6.33	50 ± 4.14

**Addition of elicitor on 2nd day*

Effect of cyano-bacterial (Nostoc carneum) elicitor on the bioamss and growth rate of cell suspension cultures of neem variety KN1

The effect of elicitor *Nostoc carneum* appears to be relatively low when compared to *Anabaena sp.* The concentration of 1670/ml *Nostoc carneum* showed optimum biomass 80gm/l on 3rd day whereas in control it was maximum (48.09gm/l) on 2nd day of culture (Table 3). However, 2.2 folds increase of biomass by indicated by growth index 4th and 5th days of neem cell suspension cultures is exclusively due to algal elicitor (Table 4). Unlike fungi algae are autotrophic, though autoclaved sample is used as elicitor has revealed remarkable enhan-cement.

Enhancement of protein content in neem callus biomass using cyanobacterial elicit -ors

Since growing cell are actively involved in protein synthesis, the protein content of neem suspension callus due to growth induction by algal elicitors, is higher than that of control. Out of the two cyanobacterial elicitors, *Anabaena* induced higher growth rates than *Nostoc carneum* which is in tune to their relative enhanced growth indices (Table 5).

Effect of cyanobacterial elicitors on the production of azadirachtin in cell suspension cultures of Neem variety KN-1

Anabaena sp. as elicitor at 265 cells/ml on neem cell suspension cultures brought out a detectable amount of azadirachtin (0.32µg/l) in the neem culture on eighth day (Table 6) while its other concentrations fail to achieve it. This is the first report where the cyanobacterial elicitors have shown positive role towards the production of tetranortriterpenoid, azadirachtin.

Discussion

Over few decades, many strategies like media manipulation, phyto-hormone regulation, precursor feeding, plant cell immobilization, biotransformation and bioconversion, hairy root cultures and genetically modified cells etc. have been tried, but failed to synthesize the desired products in appreciable quantity and at competitive economic value (Namdeo, 2007). Hence, elicitor-based enhancement of secondary metabolism in

plants or plant cells *in vitro* provides an excellent opportunity for intensive research in the exploitation of plant cells for the production of secondary metabolites.

Table 4. Effect of cyanobacterial (Nostoc carneum) elicitor on the growth rate of cell suspension cultures of neem variety KN1

Parameter	Average growth rate	Specific growth rate (Duration in days)					Growth index
		2	3	4	5	7	
Control	0.478	0.336	0.203	0.147	0.094	0.094	0.956
1670cells/ ml elicitor*	0.860	0.438	0.389	0.197	0.197	0.099	2.200

**Addition of elicitor on 2nd day*

Cyanobacterial elicitor i.e. *Anabaena sp*. can be a potential source increasing the neem callus biomass to five folds which otherwise difficult to achieve. Elicitor concentration 265 cells/ml of Anabaena *sp.* revealed 0.32 µ\l azadirachtin on 8th day during the log phase. Unlike fungi, algae are autotrophic, though autoclaved sample is used as elicitor it has revealed remarkable enhancement on growth. However, further studies on type and concentration of algal elicitor may yield positive results on AZ production.

Table 5. Enhancement of protein content in neem callus biomass using cyanobacterial elicitors

Sample		Protein content mg/ml
Without elicitor	Control	6
Cyanobacterial elicitor	*Nostoc carneum*	13
	Anabaena sp.	19

Production of azadirachtin from suspension cultures of *Azadirachta indica* using fungal elicitor had been attempted by quite a number of investigators (Satdive *et al.,* 2007; Sevon *et al*., 2002). Elictors of both fungal and bacterial origin that is polysacccharides, proteins and fatty acids are widely used for enhancement of secondary metabolites in plant cell cultures.

Variety of microbial components is to known to act as elicitors. Bacterial lipopolysaccharides could be strong triggers of early events of defense reactions in Brown algal kelp *Laminaria digitata* constitute the first report of a biological activity of this class of macro molecules in a marine algae. The major cell wall polysaccharides being the alginate would react with the oxidative burst Lamouroux, (in *Laminaria sp*.) had been shown to play a crucial role in controlling the growth of epiphytic and pathogenic bacteria producing hydrogen peroxide to reduce the bacterial growth and to reduce the number of microorganisms on algal surfaces (Kupper *et al*., 2006). A lone investigation reporting on marine red agarophyte *Gracilaria conferta* (Weinberger and Friedlander, 2000) indicated a low molecular weight (700-1500 DA) peptide was responsible as elicitors.

The major light harvesting pigment, phycocyanin of *Spirulina platensis* was used as an elicitor to enhance the accumulation of capsaicin and anthocyanin in *Capsicum frutescens and Daucas carota* cell cultures respectively. Two-fold increase of capsaicin in *Capsicum frutescens* and of anthocyanin in *Daucas carota* was obtained. Phycocyanin showed an early elicitation of secondary metabolites (Rao *et al.,* 1996).

Early events in the perception of liposaccharides in the brown alga, *Laminaria digitata* include an oxidative burst and activation of fatty acid oxidation cascades. Oligosaccharide elicitors may be good candidates for a new type farm chemical for disease control. They are natural products and recyclable in the ecosystem. The laminarin polysaccharide of *Laminaria* when treated for tobacco plants showed resistance against pathogenic bacteria *Erwinia cartovora* which causes soft rot disease. Laminarin similarly induced defense responses in suspension cultured- grape vine cells and conferred resistance against pathogenic fungi *Plasmopora viticola,* which is causative agent of powdery mildew disease (Aziz *et al.,*

Table 6. Effect of cyanobacterial elicitors on the production of azadirachtin in cell suspension cultures of neem variety KN-1

Parameter	Amount of azadirachtin (µg/l)				
	2nd day	4th day	6th day	8th day	10th day
Control	nil	nil	nil	nil	nil
265 cells/ml Elicitor*	nil	nil	nil	0.32	nil
530 cells/ml Elicitor*	nil	nil	nil	nil	nil
1670 cells/ml Elicitor**	nil	nil	nil	nil	nil

** Anabaena sp.*; ***Nostoc carneum*

2003). Oligomer of sulphated fucan of *Fucus* brown marine alga was shown to induce defense response in Tobacco against Tobacco mosaic virus (Klarzynski *et al.,* 2003).

Owing to the medicinal, pharmaceutical and agricultural importance of *Azadirachta indica*, the present study creates a new avenue for enhancement of bio-pesticide azadirachtin production using particularly algal/cyanobacterial elicitors.

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