J. Plant Biotechnology(2003) Vol. 5(2). pp. 121~129

Journal of Plant Biotechnology

Azadirachtin, a Novel Biopesticide from Cell Cultures of *Azadirachta indica*

Kaveti Balaji, Ciddi Veeresham*, Keshetty Srisilam, Chandrakanth Kokate

Faculty of Pharmaceutical Sciences, Kakatiya University, Warangal 506009, India

Abstract

Callus cultures of *Azadirachta indica* flower petals were established on MS medium supplemented with naphthalene acetic acid (1 mg/L), kinetin (0.5 mg/L) and sucrose (3% w/v). Cell cultures of *Azadirachta indica* were established and studied the growth and production kinetics. Half B5 medium supplemented with dicamba (2 mg/L), kinetin (1 mg/L) and sucrose (3% w/v) was found to be suitable for initiation and maintenance of cell cultures from the calli. MS medium supplemented with naphthalene acetic acid (NAA) (1 mg/L), kinetin (0.5 mg/L) and sucrose (3% w/v) was found to be suitable as production medium. Around 80% (0.05% w/v) of azadirachtin was found to be intracellular. The effect of various precursors, elicitors, permeabilizing agents and growth retardants in cell cultures was studied. The addition of precursors sodium acetate (10 mg/L), squalene (10 mg/L), isopentenyl pyrophosphate (1 mg/L) and geranyl pyrophosphate (1 mg/L) to the cell cultures on day 3 has shown significant increase in bioproduction of azadirachtin (64.94 $\pm$ 4.40 mg/L, 72.81 $\pm$ 0.04 mg/L, 51.63 $\pm$ 1.26 mg/L and 30.70 $\pm$ 0.28 mg/L respectively) over the control cultures (4.70 $\pm$ 0.27 mg/L). 5% v/v cell extracts of *Fusarium solani* has shown moderate increase in the content of azadirachtin (5.71 $\pm$ 0.34 mg/L) when compared to control cultures (2.40 $\pm$ 0.56 mg/L). The addition of methyl jasmonate (500 μM/L) on day 3 has shown ~4 fold improvement in bioproduction of azadirachtin (6.92 $\pm$ 0.11 mg/L) when compared to control cultures (1.63 $\pm$ 0.02 mg/L). There was no significant effect of the studied growth retardants and permeabilizing agents on bioproduction of azadirachtin. Cells are cultivated in large volumes using the effective precursors.

* Corresponding author, E-mail: ciddiveeresham@yahoo.co.in
Received Feb. 10, 2003; accepted Apr. 5, 2003

Key words: *Azadirachta indica*, cell cultures, elicitors, growth retardants, permeabilizing agents, precursors

Introduction

Azadirachtin a tetranortriterpenoid first isolated by Butterworth and Morgan (1968) from the seed of *Azadirachta indica* A. Juss; Family: Meliaceae and it is used a natural biopesticide. There is much current interest in its use as a natural pesticide because of its eco-friendly in nature. Azadirachtin is a high cost biochemical; the complexity of structure precludes synthetic production system. Moreover, current supplies of bioactive compounds from the neem tree will not meet the increasing demands (Saxena 1989). These factors suggest that neem cell cultures may act as an alternative method for the production of azadirachtin. There are several reports on the callus cultures of *A. indica* for the bioproduction of azadirachtin (Wetzer 1994; Allan et al. 1994; Srividya et al. 1998). Veeresham et al. (1998) reported that the 12-week-old flower callus produced 2.46% (on dry wt. basis) of azadirachtin. However, the yields are very low. The amount of azadirachtin bioproduction in callus and cell cultures (0.006% & 0.05% respectively on dry wt. basis) was comparatively lower than that reported in seeds (0.40% on dry wt. basis) (Balaji 2001). These results suggested that there is a scope to adopt certain yield improvement strategies like addition of precursors, elicitors (biotic and abiotic), permeabilizing agents and growth retardants to the cell cultures to enhance the productivity of azadirachtin. Ballica et al. (1993) reported that yield of tropane alkaloid was five times higher in *Datura stramonium* cell cultures supplemented with precursors L-phenylalanine and L-ornithine as compared to that of control cultures. Veeresham et al. (1995) reported that the addition of biotic elicitors, cell extracts and culture filtrates of *Pencillium minioluteum*, *Botrytis cinerea*, *Verticillium dahliae* and *Gliocladium deliquescens* improved the production of taxol

and related taxanes in *Taxus* species cell cultures. Methyl jasmonate has been shown to enhance the production of taxol and its analogues, rosmarinic acid, indole alkaloids, anthocyanins etc. in cell cultures of *Taxus* sp., *Lithospermum erythrorhizon, Catharanthus roseus* and *Vaccinium pahale* (Yukimune et al. 1996). Heijden van der et al. (1988) induced triterpenoid coronarine biosynthesis in cell suspension cultures of *Tabernaemontana* species by the addition of cellulase and pectinase. Contin et al. (1999) reported that increased tryptamine content in cell cultures of *Catharanthus roseus* was observed by the addition of pectinase. Kuruvilla et al. (1999) found enhanced secretion of azadirachtin by permeabilizing agents Triton × 100, DMSO, cetrimide and chitosan in cell cultures of *A. indica.* Strobel et al. (1994) reported that growth retardants chlorocholine chloride (CCC), succinic acid, 2,2-dimethyl hydrazide and tetra methyl ammonium bromide (TMAB) stimulate taxol synthesis in intact pieces of the inner bark of *Taxus brevifolia.* Jose et al. (1987) reported that sterol inhibitor miconazole and CCC increase both the incorporation of ^{14}C-isopentenyl pyrophosphate into artemisinin by cell free extracts and production of artemisinin in shoot cultures of *Artemisia annua.*

In present investigation, we report the influence of precursors, sodium acetate (SA), squalene (SQ), cholesterol, isopentenyl pyrophosphate (IPP), geranyl pyrophosphate (GPP) and dimethyl allyl pyrophosphate (DMAP); biotic elicitors, cell extracts and culture filtrates of *Alternaria alternata, Fusarium solani* and *Verticillium dahliae*; abiotic elicitors, methyl jasmonate (MJ), copper sulfate, salicylic acid, cellulase, pectinase, pectolyase and silver nitrate; long term permeabilization using chitosan, Triton × 100, DMSO and cetrimide and growth retardants, CCC and ancymidol on bioproduction of azadirachtin in cell cultures of *A. indica.*

Materials and Methods

Initiation and maintenance of callus

The callus cultures were initiated from flower petals collected during March-April 2000 from Kakatiya University Campus, Warangal, India. The flower petals were surface sterilized by treatment with mercuric chloride (0.1% w/v) for 5 minutes and transferred to Murashige & Skoog medium supplemented with NAA (1 mg/L), kinetin (0.5 mg/L) and sucrose (3% w/v). The callus cultures were maintained on the same medium.

Initiation of suspension cultures of *A. indica*

The fragile 8-week old callus (2 g) was aseptically transferred into 0.5 B5 medium (40 mL) supplemented with dicamba (2 mg/L), kinetin (1 mg/L) and sucrose (3% w/v) without agar. The cultures were incubated at 26°C ± 2°C and 120 rpm in shaker incubator.

Growth kinetics

From the suspension cultures growing in growth medium, the aliquots were withdrawn at the end of 3rd, 5th, 7th, 10th and 15th day of incubation. Cultures were filtered and the cells were weighed for determination of growth of the cells. This procedure was repeated thrice and the growth indices (G.I) were calculated.

Production kinetics

The cell cultures (in growth medium) after two passages, were cultured into the production medium [MS medium supplemented with NAA (1 mg/L), kinetin (0.5 mg/L) and sucrose (3% w/v) with 50% v/v as inoculum] so as to induce production of azadirachtin. The cell cultures (10 mL) were aseptically transferred into each of pre-sterilized conical flasks (50 mL capacity) with a sterilized pipette (10 mL capacity) under laminar flow and incubated at 26°C ± 2°C and 120 rpm in shaker incubator. The cultures were withdrawn at the end of 3rd, 5th, 7th, 10th, 15th and 20th day, extracted and analyzed for azadirachtin content.

Addition of precursors, elicitors, permeabilizing agents and growth retardants

After 4 passages in growth medium the cell cultures were transferred into production medium with 50% v/v as inoculum. 10 mL of cell cultures was aseptically transferred into each of the presterilized conical flasks (50 mL capacity) with a sterilized pipette (10 mL capacity).

The precursor solutions sodium acetate (10 mg/L and 100 mg/L), squalene (10 mg/L and 100 mg/L), cholesterol (10 mg/L and 100 mg/L), isopentenyl pyrophosphate (1 mg/L and 3 mg/L), geranyl pyrophosphate (1 mg/L and 3 mg/L) and dimethyl allyl pyrophosphate (1 mg/L and 3 mg/L); the biotic elicitor solutions, 1% and 5% v/v of fungal cell extracts (CE) or culture filtrates (CF) of *Alternaria alternata / Verticillium dahliae / Fusarium solani*; the abiotic elicitors, MJ (100 μM and 500 μM), copper sulfate (100 μM and 500 μM), salicylic acid (100 μM and 500 μM), cellulase (0.1% and 0.5% w/v), pectinase (0.01% and 0.05% w/v), pectolyase (0.01% and 0.05% w/v) and silver nitrate (0.1 mg/L and 1 mg/L); the permeabilizing agents, DMSO (0.1% v/v and 1% v/v), Triton × 100 (100 ppm and 500 ppm), cetrimide (25 ppm and 50 ppm) and chitosan (0.5 mg/L and 3 mg/L); and the growth retardants ancymidol (10 μM/L and 100 μM/L) and CCC (0.1 mM/L and 1 mM/L) with suitable controls were added on day-0 to the cell cultures in

each of the culture flask under laminar flow and incubated at 26°C±2°C and 120 rpm. Similarly the effective precursors and elicitors were further studied for optimum day of addition (day 0, 3, 5 and 7). For each concentration, three culture flasks were used, while running suitable control. The cultures were withdrawn at the end of day-10, extracted both the cells and media together for azadirachtin and analyzed.

Extraction of azadirachtin

The cultures after 10 days in production were extracted for azadirachtin. The cells and media together were extracted with methanol (3×10 mL). To the methanolic extract, equal volume of distilled water was added and partitioned with dichloromethane (3×10 mL). The dichloromethane layers were pooled together and concentrated under vacuum. The residue was dissolved in 1 mL methanol (HPLC grade). This solution was subjected to TLC and HPLC analysis (Yamasaki et al. 1986).

Analysis

Thin Layer Chromatography (TLC)

The methanolic extracts were co-chromatographed on precoated aluminum silica gel-G (Merck) plates with authentic sample using hexane-solvent ether (2:8) as solvent system. Vanillin-sulphuric acid was used as detecting system.

High-Performance Liquid Chromatography (HPLC)

After detection of azadirachtin by TLC, HPLC analysis was carried on Shimadzu LC 10AT system, by injecting 10 μL of the each standard solution and extract with Hamilton syringe, using C-18 column (SGE, Wakosil-II Analitica, 250×4.6 mm i.d., 5 μL) with Shimadzu Photo Diode Array (SPD-M10 AVP model) detector. The mobile phase (acetonitrile-water 40:60) was pumped isocratically at a flow rate of 1 mL/min and the azadirachtin was detected at 217 nm (Yamasaki et al. 1986; Thejavathi et al. 1995).

Results and Discussion

The callus cultures of *Azadirachta indica* were initiated and maintained on MS medium supplemented with NAA (1 mg/L), kinetin (0.5 mg/L) and sucrose (3% w/v). The cell cultures were initiated from 8-week old callus cultures. 0.5 B5 medium supplemented with dicamba (2 mg/L), kinetin (1 mg/L) and sucrose (3% w/v) was found to be suitable for initiation and maintenance of cell cultures from the calli. The growth kinetics of cell cultures is depicted in Figure 1. The maximum growth of the cells was found to be on day 10. A decline in growth was observed on day 15. MS medium supplemented with sucrose (3% w/v), NAA (1 mg/L) and kinetin (0.5 mg/L) was found to be suitable as production medium. The production kinetics of azadirachtin in cell cultures is shown in Figure 2. The maximum amount of azadirachtin was found to be 10.11±2.41 mg/L on day 7 using production medium. On co-chromatography with authentic sample, the extracts obtained from the cultures revealed the presence of azadirachtin. The extracted samples has the same retention factor (R_f) (0.28) value as that of the authentic sample and shown a violet-black spot upon treatment with vanillin - sulphuric acid. On HPLC analysis, the extracts obtained from cell cultures had the same retention time (R_t) values as that of authentic sample (15.6 min). Further, the extracts obtained from cell cultures had the same UV absorption maxima as that of authentic sample in Diodearray detector. The production profile of azadirachtin revealed that the maximum bioproduction of azadirachtin was on day 7 using production medium. However, there was a decrease in the production of azadirachtin with the increased period of incubation. The content of azadirachtin in cells and media was found to be 9.31 ±2.40 mg/L and 0.80±0.001 mg/L respectively. These results

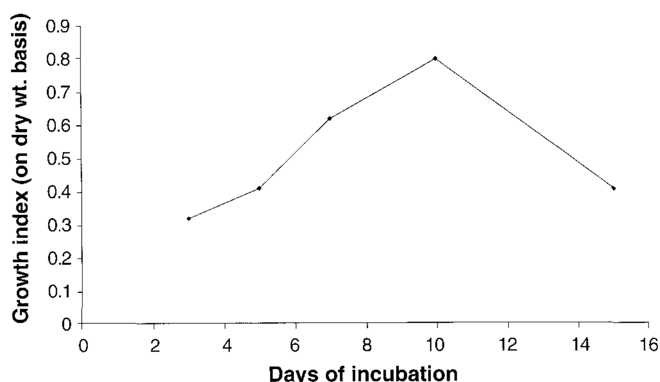

Figure 1. Growth Kinetics of Cell Suspension Cultures of *Azadirachta indica*

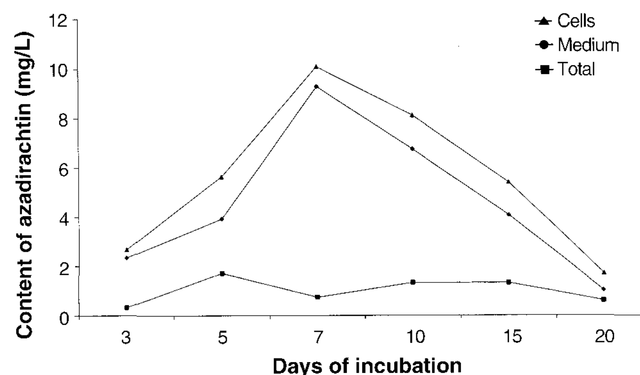

Figure 2. Production Kinetics of Azadirachtin in Suspension Cultures of *Azadirachta indica*

suggests that majority of the azadirachtin was produced as intracellular. The comparative profile of azadirachtin production in intact plant, callus and cell cultures of *A. indica* is shown in Figure 3. The content of bioproduction of azadirachtin in cell cultures is almost 10 folds more than in callus cultures derived from different explants. However, the yield of azadirachtin in cell cultures was one tenth to that reported in seeds (0.4% on dry wt. basis).

The addition of sodium acetate (10 mg/L) to the cell cultures of *A. indica* significantly improved the production of azadirachtin over the control cultures. There was ~ 8-fold increase in azadirachtin content by supplementation of sodium acetate (10 mg/L) to the cell cultures (30.72±2.56 mg/L) over the control cultures (3.92± 0.72 mg/L) (Figure 4). The overall improvement in the yield of azadirachtin may be due to its incorporation as a precursor for the biosynthesis of azadirachtin. It was reported that azadirachtin belongs to the C-seco-limonoid group of triterpenoids, and is biogenetically formed from acetate via mevalonate, squalene, apo-tirucallol and subsequent oxidation (Butterworth and Morgan 1968; Ley et al. 1993). The earlier reports suggest that proto-meliacins or proto-limonoids (euphane or triucallane derivatives) are the biosynthetic precursors of meliacins or limonoids (tetranortetracyclic triterpenoids) (Lavie and Levy 1971).

The addition of squalene (10 mg/L) to the cell cultures of *A. indica* significantly improved the production of azadirachtin. There was ~11-fold increase in azadirachtin bioproduction by supplementation of squalene (10 mg/L) to the cell cultures (27. 38±0.014 mg/L) over the control cultures (2.43±0.28 mg/L) (Figure 4). The overall improvement of azadirachtin may be

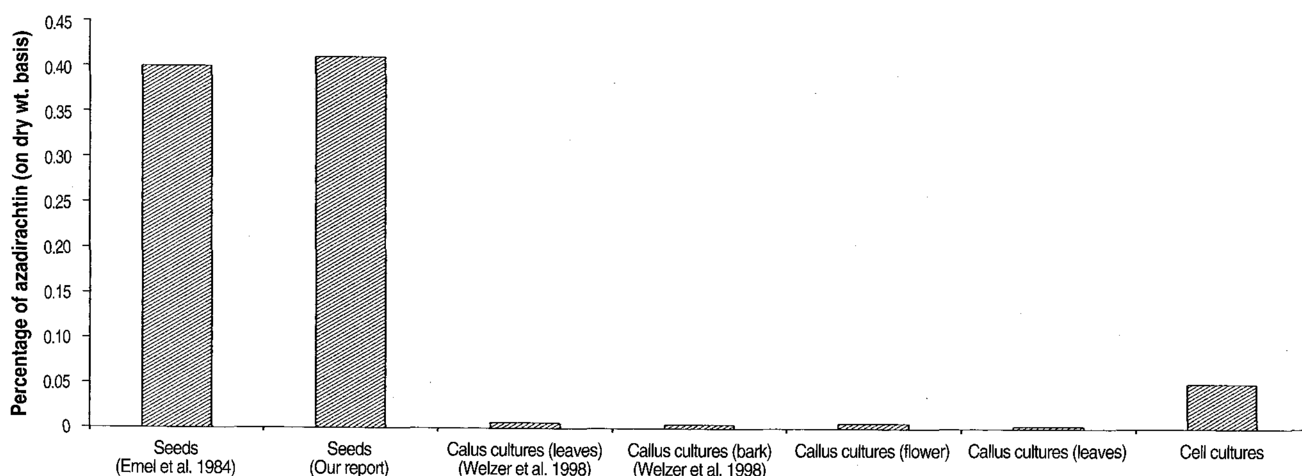

Figure 3. Comparative statement of azadirachtin content from different source (Intact & *In vitro* Cultures)

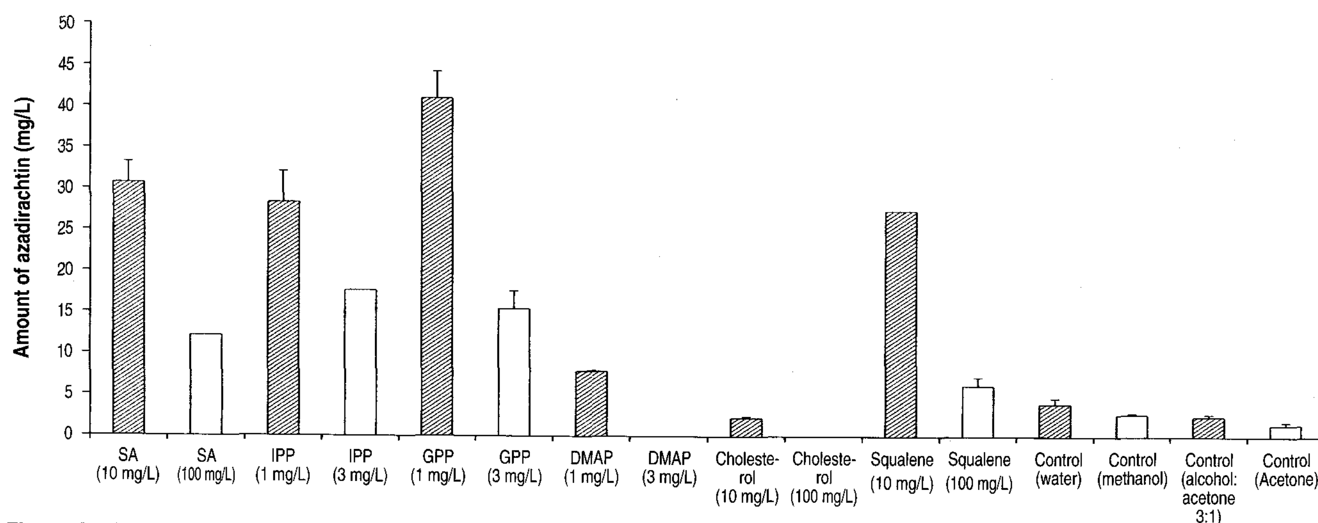

Figure 4. Effect of various precursors on the bioproduction of azadirachtin in cell cultures of *A. indica*

due to precursor incorporation in its biosynthesis. Ekong et al. (1985) reported that the biosynthetic pathway of limonoids involves the incorporation of squalene for bioproduction of euphol or butyrospermol, which are intermediates in azadirachtin biosynthesis in intact plant.

The addition of isopentenyl pyrophosphate (1 mg/L) to the cell cultures of *A. indica* significantly improved the production of azadirachtin (28.33±3.80 mg/L) over the control cultures (2.65 ±0.18 mg/L) (Figure 4). There was ~ 11-fold increase in azadirachtin bio-production by supplementation with isopentenyl pyrophosphate (1 mg/L) to the cell cultures observed over the control cell cultures. The azadirachtin is biosynthesized through acetate-mevalonate pathway. The isopentenyl pyrophosphate is one of the key intermediates in acetate-mevalonate pathway. Hence, its enhanced effect on bioproduction may be due to increased availability of precursor for the biogenesis of azadirachtin (Newmann and Chappell 1999).

Addition of geranyl pyrophosphate (1 mg/L) to the cell cultures of *A. indica* also enhanced the yield of azadirachtin (41.05±3.19 mg/L) as compared to the control cultures (2.65 ±0.18 mg/L). There was ~16-fold increase in azadirachtin production by supplementation of geranyl pyrophosphate (1 mg/L) to the cell cultures over the control cultures (Figure 4). The overall improvement of azadirachtin, by addition of geranyl pyrophosphate in the cell cultures of *A. indica* may be due to its incorporation as a precursor in biosynthesis of azadirachtin.

The addition of cholesterol (10 mg/L) to the cell cultures of *A. indica* has not shown significant effect on bioproduction of azadirachtin (Figure 4). In earlier reports, the addition of cholesterol has shown enhancement in solasodine content in suspension cultures of *Solanum xanthocarpum* and *Solanum aviculare* (Khanna et al. 1976; Bhargava 1979). In our study cholesterol failed to enhance the bioproduction of azadirachtin may be due to its preference towards the biosynthesis of sterols than terpenoids or it may not be reaching the site of action.

The addition of dimethyl allyl pyrophosphate (1 mg/L) to the cell cultures of *A. indica* improved the production of azadirachtin (7.84±0.19 mg/L) over the control cultures (2.65±0.18 mg/L) (Figure 4). There was approximately 3 fold increase in azadirachtin production by supplementation of dimethyl allyl pyrophosphate to the cell cultures over the control. The lower dose of the precursor was found optimum to induce bioproduction of azadirachtin. The improvement in bioproduction of azadirachtin indicates the incorporation of this precursor in biosynthesis of azadirachtin.

All precursors in higher concentration showed less effect on enhancement of azadirachtin bioproduction in cell cultures of *A. indica*. It may be due to negative feed back mechanism in biogenetic pathway.

The precursors, which were found effective, were further studied for determination of optimum day of their addition. On addition of sodium acetate (10 mg/L) on days 0, 3, 5 and 7 has shown about 6.5, 16, 11 and 7 folds increase respectively in the content of azadirachtin (27.38±0.014 mg/L, 64.94±4.40 mg/L, 47.21±2.90 mg/L and 28.82±3.32 mg/L respectively) over the control cultures (4.16±2.54 mg/L) (Figure 5). The addition of sodium acetate (10 mg/L) on day 3 was found to show more significant effect than the additions on days 0, 5 and 7. The addition of squalene (10 mg/L) on days 0, 3, 5 and 7 has shown 6.5, 16, 11 and 5 folds increase in content of azadirachtin [30.72±1.53 mg/L, 72.81±0.04 mg/L, 50.4±0.12 mg/L and 21.74 mg/L respectively over control cultures (4.70±0.27 mg/L)] (Figure 6). Upon addition of isopentenyl pyrophosphate (1 mg/L) and geranyl pyrophosphate (1 mg/L) on day 3, 14 and 8 folds increase respectively in amount of azadirachtin could be detected (Figure 7 and 8).

All the precursors studied for optimization of day of addition have shown appreciable enhancement in yield of azadirachtin

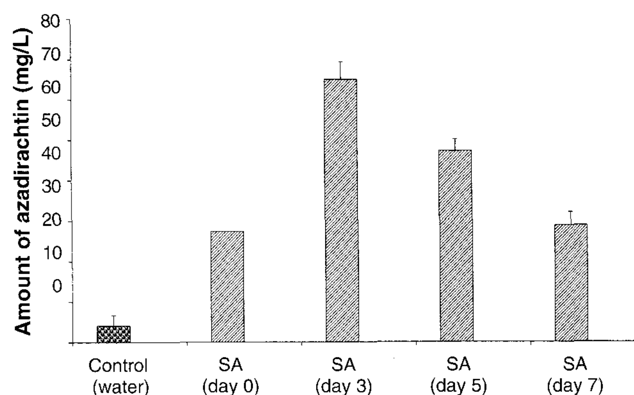

Figure 5. Effect of sodium acetate on different days of addition to the cell cultures of *A. indica*

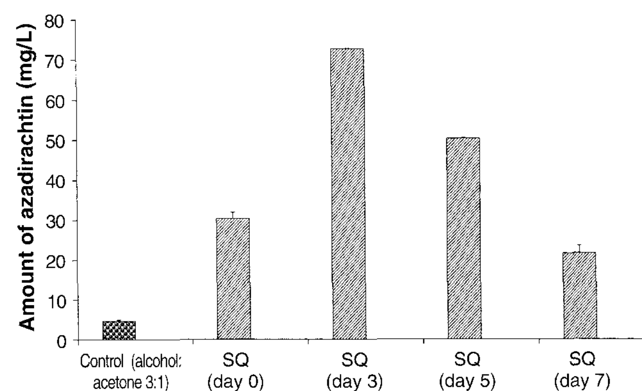

Figure 6. Effect of squalene on different days of addition to the cell cultures of *A. indica*

upon day-3 addition. This could be due to the reason that the biogenetic process is positively accelerated leading to increased production of azadirachtin.

There is no significant effect of cell extracts and culture filtrates on *Alternaria alternata, Verticillium dahliae* and *Fusarium solani* on the bioproduction of azadirachtin in cell cultures of *A. indica* except with the cell extracts of *F. solani* (Figure 9). The addition of cell extract (5% v/v) of *F. solani* showed 2-fold improvement in bioproduction of azadirachtin (5.71±0.34 mg/L) over control cultures (2.40±0.56 mg/L). Whereas in case of *A. alternata* and *V. dahliae*, the addition of culture filtrates has shown 1.3 and 1.5 fold improvement in bioproduction of azadirachtin (3.18±1.54 mg/L and 3.54±0.07 mg/L respectively) over control cultures. However, the cell extracts of *F. solani* and culture filtrates of *A. alternata* and *V. dahliae* were found to show significant effect only in higher concentration (5% v/v) on bioproduction of azadirachtin in cell cultures of *A. indica*. It may due to the fact that fungal extracts used may have sufficient amount of compounds only in higher concentration, capable of stimulating the plant defensive system in present set of experiment or it might be the sufficient concentration to reach the appropriate site of action to enhance the yield of secondary metabolites.

The addition of methyl jasmonate (MJ) (500 μM/L) on 0 day has shown ~3 fold improvement in production of azadirachtin (5.84±0.71 mg/L) over the control cell cultures (2.24±0.13 mg/L) (Figure 10). There was no significant effect of MJ (100 μM/L) on day 0 addition, on the production of azadirachtin, compared to control cell cultures.

The addition of copper sulphate (100 μM/L and 500 μM/L) on day-0 had no significant effect on the production of azadirachtin (2.82±0.35 and 1.12±0.15 mg/L respectively) over the control cultures (1.98±0.84 mg/L) (Figure 10). It may be due to its inability to induce the enzymes involved in biogenetic pathway of azadirachtin or the elicitor may not be reaching the site of action or toxic to the cells. It was reported that, copper (II) ions were optimal for inducing the accumulation of high levels of sesquiterpenoid phytoalexins (lubimin, 3-hydroxy lubimin

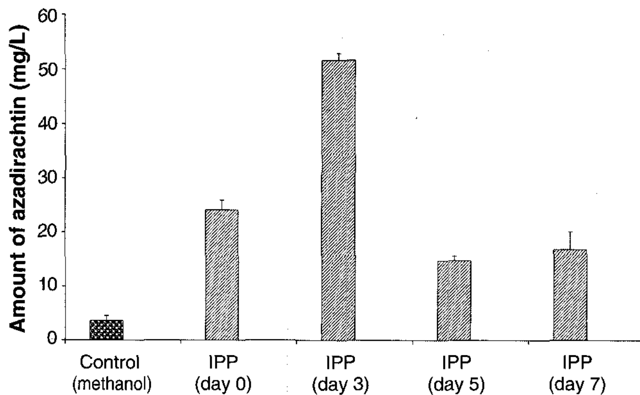

Figure 7. Effect of isopentenyl pyrophosphate on different days of addition to the cell cultures of *A. indica*

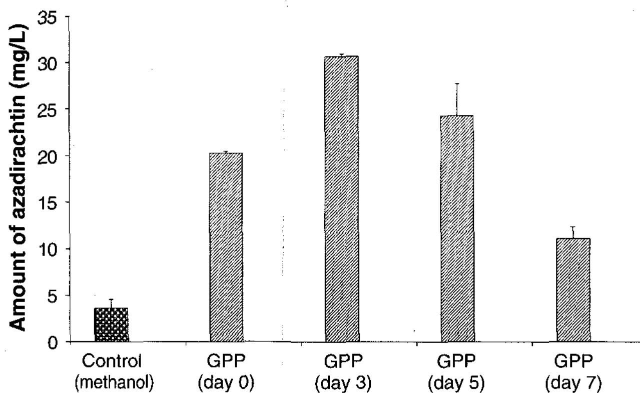

Figure 8. Effect of geranyl pyrophosphate on different days of addition to the cell cultures of *A. indica*

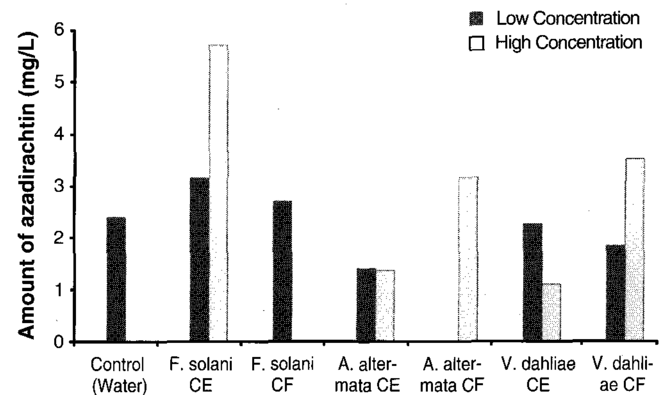

Figure 9. Effect of biotic elicitors on bioproduction of azadirachtin in cell cultures of *Azadirachta indica*

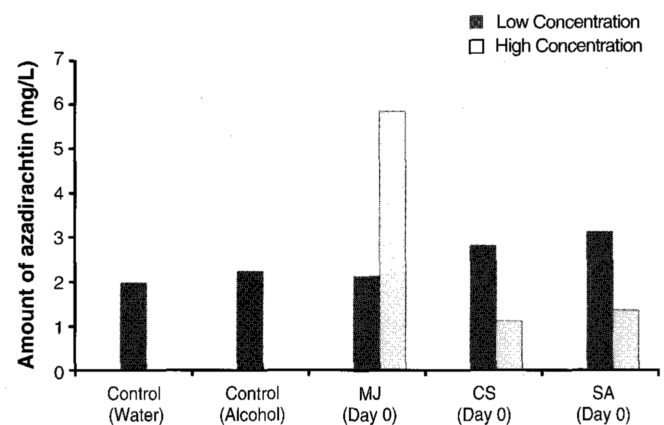

Figure 10. Effect of elicitors on bioproduction of azadirachtin in cell cultures of *Azadirachta indica*

and rishitin) in fruit cavities of thorn apple (*Datura stramonium*) whilst, in cell suspension cultures, the highest levels of product were formed in response to 1 mM copper (II) ions (Whitehead et al. 1990).

The addition of salicylic acid (100 μM/L and 500 μM/L) on day-0 had no significant effect on the production of azadirachtin (3.12±0.11 and 1.36±0.43 mg/L respectively) over the control cultures (1.98±0.84 mg/L) (Figure 10). It may be due to its inability to induce the enzymes involved in biogenetic pathway of azadirachtin or the elicitor may not be reaching the site of action or toxic to the cells.

The addition of cellulase (0.1% and 0.5% w/v), pectinase (0.01% and 0.05% w/v), pectolyase (0.01% and 0.05% w/v) and silver nitrate (0.1 mg/L and 1 mg/L) on day-0 had no significant effect on the production of azadirachtin (17.58±1.65 and 27.48±5.60 mg/L, 14.53±1.88 and 25.70±4.70 mg/L, 15.34 ±0.64 and 26.27±0.68 mg/L and 46.49±6.46 and 90.19± 10.78 mg/L respectively) over the control cultures (78.84± 16.27 mg/L) (Figure 11). It may be due to its inability to induce the enzymes involved in biogenetic pathway of azadirachtin or the elicitor may not be reaching the site of action or toxic to the cells.

The elicitors, which were found effective, were further studied for determination of optimum day of their addition. There was a significant improvement (4 fold) in bioproduction of azadirachtin (6.92±0.11 mg/L) over control cultures (1.63±0.02 mg/L) observed on day 3 addition of methy jasmonate (500 μM/L) (Figure 12). These results indicated that the optimum day of addition of methy jasmonate (MJ) was day 3.

In conclusion, the methyl jasmonate showed great influence on bioproduction of azadirachtin in cell cultures of *A. indica* over other elicitors employed. This is the first report of the effect of methyl jasmonate on the production of azadirachtin in cell cultures of *Azadirachta indica*. Methyl jasmonate is thought to be the precursor of the jasmonic acid which is one of the intermediates involved in the signal transduction.

The effect of long term permeabilization using DMSO, Triton ×100, cetrimide and chitosan on the bioproduction of azadirachtin in cell cultures of *Azadirachta indica* is shown in Figure 13. The addition of DMSO (0.1% and 1% v/v), cetrimide (25 ppm and 50 ppm) and Triton × 100 (100 ppm and 500 ppm) on day-0 had no significant effect on the production of azadirachtin (30.81 ± 3.45 and 63.45 ± 6.24 mg/L; 10.06 ± 0.30 and 10.93 ± 1.30 mg/L and 5.20 ± 0.005 mg/L and 25.32 ± 14.17 mg/L respectively) over the control cultures (25.65±6.55 mg/L). Park and Martinez (1992) reported 66% release of rosmarininc acid from *Coleus blumei* cell cultures using DMSO as permeabilizing agent at 1% v/v concentration. Brodelius (1988) reported that cetrimide in the concentrations of 44 and 72 ppm in cell cultures of *Catharanthus roseus* enhanced the release of indole alkaloids by 50%. Brodelius et al. (1988) reported 50% release of alkaloids from cultured cells of *Catharanthus roseus* using Triton × 100 as permeabilizing agent. Similar effect was not observed in case of *Azadirachta indica* cell cultures.

Both the doses of chitosan (0.5 mg/L and 3 mg/L) employed inhibited the production of azadirachtin (53.40±8.06 and 20.30 ±6.36 mg/L respectively) when compared with control cell cultures (66.34±8.79 mg/L). Chitosan (0.5 mg/L) showed 10.5% decrease and at concentration 3 mg/L showed 69.4% decrease in the content of azadirachtin. Kuruvilla et al. (1999) reported that permeabilizing agents DMSO, chitosan and Triton × 100 enhanced the secretion of azadirachtin from the callus culture of *Azadirachta indica*. Their results also indicating that enhanced release of azadirachtin by permeabilizing agents also enhanced the overall productivity of azadirachtin when compared with untreated cultures. The maximum amount of azadirachtin reported was found to be 10 mg/L. However, in our study all the permeabilizing agents employed was not

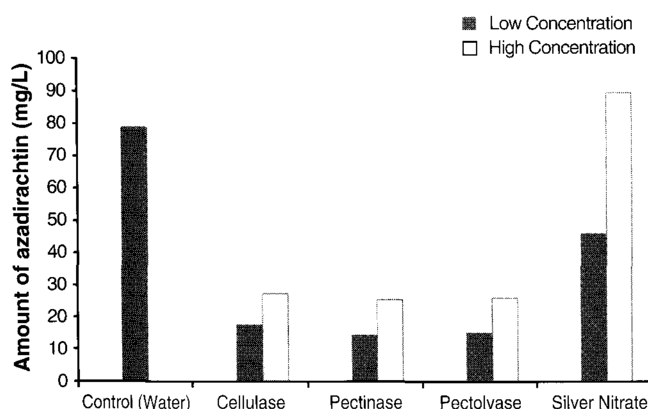

Figure 11. Effect of elicitors on bioproduction of azadirachtin in cell cultures of *Azadirachta indica*

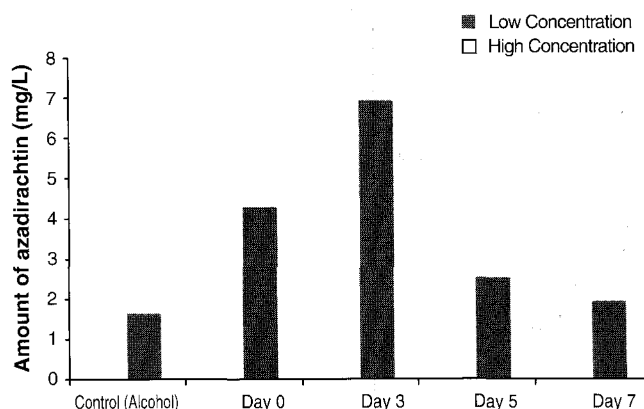

Figure 12. Influence of day of addition (Methyl jasmonate, 500 μM/L) on bioproduction of azadirachtin in cell cultures of *Azadirachta indica*

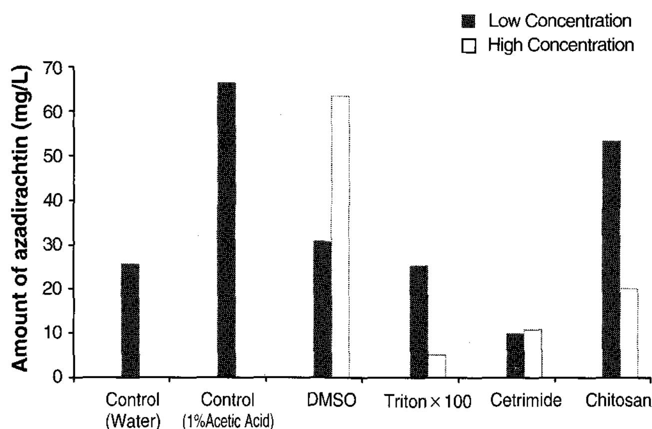

Figure 13. Effect of permeabilizing agents on bioproduction of azadirachtin in cell cultures of *Azadirachta indica*

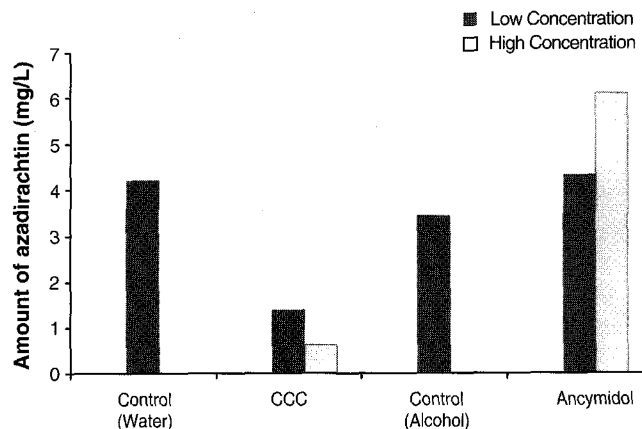

Figure 14. Effect of growth retardants on bioproduction of azadirachtin in cell cultures of *Azadirachta indica*

enhanced the production of azadirachtin. The maximum amount of azadirachtin in our study was 90.19±10.78 mg/L. It seems that the enhancement of azadirachtin by permeabilizing agents also depends on the productivity of the culture. The culture employed in the present study shown 9 fold increased productivity compared to the cell cultures of Kuruvilla et al. (1999).

The effect of addition of growth retardants on bioproduction of azadirachtin in cell cultures has shown in Figure 14. The addition of chlorocholine chloride (CCC) (0.1 mM and 1 mM) to the cell cultures could not enhance the production of azadirachtin (1.40±0.32 mg/L and 0.63±0.20 mg/L respectively) over the control cultures (4.20±0.01 mg/L). The CCC inhibits the incorporation of farnesyl pyrophosphate into sterol.

There was no significant effect on addition of ancymidol (10 μM/L and 100 μM/L) on the bioproduction of azadirachtin in cell cultures (4.32±2.21 mg/L and 6.10±0.24 mg/L) over the control cultures (3.44±0.50 mg/L).

In conclusion, the precursors enhanced the productivity of azadirachtin in cell cultures of *Azadirachta indica*. However, there is a need to study further employing with the radiolabeled precursors to show the effect. The methyl jasmonate had a significant effect on the production of azadirachtin, while growth retardants and permaeabilizing agents had no significant effect. The effect of various precursors, elicitors, permeabilizing agents and growth retardants may depend on cell line productivity. The maximum productivity of the cell line was observed at 90 mg/L of azadirachtin. The productivity of the culture varied from 1.63±0.02 mg/L to 90.19±10.78 mg/L throughout the study.

Acknowledgements

The authors are thankful to Department of Biotechnology (DBT), New Delhi for the financial assistance provided during the project.

References

Allan EJ, Easwara JP, Shaun J, Jennifer MA, David ME, Trevor S (1994) The production of azadirachtin by *in vitro* tissue cultures of neem, *Azadirachta indica*. Pest Sci 42: 147-152

Balaji K (2001) Studies on tissue cultures of *Azadirachta indica* (neem), Ph.D. Thesis submitted to Kakatiya University, Warangal, A.P., India

Ballica R, Ryu DDY, Kado CI (1993) Tropane alkaloid production in *Datura stramonium* suspension cultures: Elicitor and precursor effects. Biotechnol Bioeng 41: 1075-1081

Bhargava C (1979) Production of primary and secondary products from *in vitro* tissue cultures of medicinal plants, Ph.D. Thesis submitted to University of Rajasthan, Jaipur, India

Brodelius P, Funk C, Shillito RD (1988) Permeabilization of cultivated plant cells by electroporation for release of intracellularly stored products. Plant Cell Rep 7: 186-188

Brodelius P (1988) Permeabilization of plant cells for release of intracellularly stored products: Viability studies. J Appl Microbiol Biotechnol 27: 561-566

Butterworth JH, Morgan ED (1968) Isolation of a substance that suppresses feeding in locust. J Chem Soc Chem Comm: 23-24

Contin A, Heijden van der R, Verpoorte R (1999) Effect of alkaloid precursor feeding and elicitation on the accumulation of secologanin in a *Catharanthus roseus* cell suspension culture. Plant Cell Tiss Org Cult 56:111-119

Ekong DEU, Samuel A, Ibiyemi (1985) Biosynthesis of nimbolide from [2-^{14}C, (4R) 4-$^{3}H_1$] mevalonic acid lactone in the leaves of *Azadirachta indica*. Phytochem 24: 2259-2260

Ermel L, Pahlich E, Schmutterer H (1984) Comparison of neem seeds from ecotypes of Asian and African origin. Proc 2nd Int Neem Conf 91-94

Heijden R van der, Verheij ER, Schripsema J, Baerheim Svendsen Verpoorte R, Harkes PAA (1988) Induction of triterpene biosynthesis by elicitors in suspension cultures of *Tabernaemontana* species. Plant Cell Rep 7: 51-54

Jose GK, Luke L, John ES (1987) Effect of sterol inhibitors on the incorporation of 14C-isopentenyl pyrophosphate into artemisinin by a cell free system from *Artemisia annua* tissue cultures and plants. Planta Med 53: 280-284

Khanna P, Aminuddin, Sharma GL, Manot SK, Rathore AK (1976) Isolation and characterization of sapogenin and solasodine from *in vitro* tissue cultures of some solanaceous plants. Indian J Exp Biol 14: 694-696

Kuruvilla T, Komaraiah P, Ramakrishna SV (1999) Enhanced secretion of Azadirachtin by permeabilized margosa (*Azadirachta indica*) cells. Indian J Exp Biol 37: 89-91

Lavie D, Levy EC, Jain MK (1971) Limonoids of biogenetic interest from *Melia azadirachta*. Tetrahedron Lett 27: 3927-3939

Ley SV, Denholm AA, Wood A (1993) The chemistry of azadirachtin, Nat Prod Rep 10: 109-157

Newman JD, Chappell J (1999) Isoprenoid biosynthesis in plants: Carbon partitioning within the cytoplasmic pathway. Crit Rev Biochem Mol Biol 34: 95-106

Park C, Martinez B (1992) Enhanced release of rosmarinic acid from *Coleus blumei* permeabilized by DMSO while preserving cell viability and growth. Biotechnol Bioeng 40: 459-464

Saxena RC (1989) Insecticides from neem. In: Arnason JT, Philogene BJR, Morand P (eds) Insecticides of Plant Origin, pp 110-135 American Chemical Society, Washington

Sri Vidya N, Poorinma SDB, Satyanarayana P (1998) Azadirachtin and nimbin content in *in vitro* cultured shoots and roots of *Azadirachta indica* A. Juss. Indian J Plant Physiol 3: 129-134

Strobel GA, Stierle A, Hess WM (1994) The stimulation of taxol production in *Taxus brevifolia* by various growth retardants. Plant Sci 101: 115-124

Thejavathi R, Shirish R, Yakkundi, Ravindranath B (1995) Determination of azadirachtin by reversed phase high performance chromatography using anisole as internal standard. J Chromatogr A 705: 374-379

Veeresham C, Rajkumar M, Sowjanya D, Kokate CK, Apte SS (1998) Production of azadirachtin from callus cultures of *Azadirachta indica*. Fitoterapia LXIX: 423-424

Veeresham C, Venkatesh S, Shuler ML (1995) Elicitation of Taxus sp. cell cultures for production of taxol. Biotechnol Lett 17: 1343-1346

Wetzer AW (1998) Callus cultures of *Azadirachta indica* and their potential for the production of azadirachtin. Phytoparasitica 26: 47-52

Whitehead MI, Atkinson LA, Threfall RD (1990) Studies on the biosynthesis and metabolism of the phytoalexin, lubimin and related compounds in *Datura stramonium* L. Planta 182: 81-88

Yamasaki RB, Klocke JA, Lee SM, Stone GA, Darlington MV (1986) Isolation and purification of azadirachtin from neem (*Azadirachta indica* A. Juss) seeds using Flash Chromatography and HPLC. J Chromatogr 356: 220-226

Yukimune Y, Tabata H, Higuchi Y, Hara Y (1996) Methyl jasmonate induced over production of paclitaxel and baccatin III in *Taxus* cell suspension cultures. Nature Biotechnol 14: 1129-1132