Azadirachtin from cell cultures of *Azadirachta indica* A. Juss.

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The natural insecticide azadirachtin is produced (930 mg/l) in cell cultures of *Azadirachta indica* grown on MS medium containing sucrose (3%), naphthalene acetic acid (1 mg/l) and kinetin (0.5 mg/l). Azadirachtin in culture extracts was identified by Co-TLC, UV and HPLC analysis. 90% of azadirachtin is retained in the cells. The addition of elicitors such as methyl jasmonate (1-500 mm/l), salacin (200-500 mm/l), arachidonic acid (100-500 mm/l) and cellulase (100 and 500 units/l) did not improve the production of azadirachtin.

Azadirachta indica A. Juss (Meliaceae), commonly known as *Neem* and is well known for its effects against insects[1]. The greatest potency of this effect is concentrated in its seeds, from which Butterworth and Morgan isolated the active substance azadirachtin[2]. It is a highly oxidized triterpenoid. Azadirachtin has antifeedant, growth regulatory and reproductive effects against insects[3]. There is no commercially viable synthetic method for the production of azadirachtin, because of its complex chemical structure. There are 8 analogs of azadirachtin which are called A, B, C, D and so on. Azadirachtin A is commonly known as azadirachtin. The plant is reported to contain salanin, nimbin and nimbidin as major constituents. The azadirachtin content in seeds varies depending upon geographical location and season. The content of azadirachtin in seeds by weight is 0.2% to 0.8%[4].

Sanyal *et al.*[5] reported the production of nimbin and b-sitosterol from callus cultures of *A. indica*. They also demonstrated that glycine improved nimbin production in callus cultures. Wewetzer[6] reported the production of azadirachtin from callus cultures of *A. indica* (African) in the concentration of 7 mg/g to 64 mg/g dry weight depending upon media and concentration of sucrose. The best medium was found to be White's media. Allan *et al.*[1] reported that callus cultures of *A. indica* from Ghanaian strain produced azadirachtin 0.0007% on dry weight basis. The callus cultures of *A. indica* (Indian variety) from flowers induced on MS medium sucrose (3%), naphthalene acetic acid (NAA) (1 mg/l) and kinetin (Kn) (0.5 mg/l) produced azadirachtin 2.46% on dry weight basis[7]. The hairy root cultures of *A. indica* produced azadirachtin in the concentration of 1.91% on dry weight basis[8]. Jarvis *et al.*[9] isolated azadirachtin from cell cultures of *A. indica* which has identical spectral characteristics of a compound isolated from seeds of neem. Kuruvilla *et al.*[10] reported that the permeabilising agents induced enhanced secretion of azadirachtin from callus cultures of *A. indica*. In the present paper the production of azadirachtin from cell cultures of *A. indica* A. Juss was reported.

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The flower explants of neem were collected from the *A. indica* tree growing in the campus of Kakatiya University, Warangal in March, 1997 and used for callus induction. The plant material was identified in the Department of Botany, Kakatiya University, Warangal.

The plant material was washed thoroughly with tap water, followed by 10% v/v of Tween 80 detergent solution. Then the explants were surface sterilized with 70% v/v ethanol for one minute, followed by 0.1% w/v mercuric chloride for 5 min. They were thoroughly washed with sterile water and aseptically transferred onto MS medium supplemented with NAA (1 mg/l) and Kn (0.5 mg/l) and maintained by subculturing onto the same medium at an interval of 4 weeks.

The cell cultures of *A. indica* were induced by transferring 10% w/v of callus into liquid 0.5 B5 medium containing sucrose (3%), dicamba (2 mg/l) and Kn (1 mg/l) by shaking at 120 rpm at 25±2° in dark. After 2 w of incubation the cell clumps were separated by filtration. Then the cultures were maintained by transferring 50% v/v inoculum into new media at an interval of 1 w. After 3 passages the cultures were

TABLE 1: EFFECT OF MEDIUM ON THE PRODUCTION OF AZADIRACHTIN

Medium	Hormones	Sugar conc. (%)	Azadirachtin (mg/l)
MS	NAA(1 mg/l) + Kn (0.5 mg/l)	3	930
MS	Dicamba (2 mg/l) + Kn (1 mg/l)	3	540
B5	Dicamba (2 mg/l) + Kn (1 mg/l)	3	320
0.5 B5	Dicamba (2 mg/l) + Kn (1 mg/l)	3	ND*
0.25 B5	2-4-D (2 mg/l) + Zeatin (0.5 mg/l)	5	Small aggregates of cells
Whites	Dicamba (2 mg/l) + Kn (1 mg/l)	3	ND*
WPM	Dicamba (2 mg/l) + Kn (1 mg/l)	3	ND*

*ND stands for not detected

transferred into different medium for the production of azadirachtin (Tables 1 and 2).

The culture broth was kept in a deep freezer for overnight and thawed, extracted with 3 volumes of dichloromethane, separated and the process was repeated 3 times for complete extraction. The combined dichloromethane layer was evaporated and the residue was dissolved in 2 ml of HPLC grade methanol. The resulting solutions were used for analysis.

The methanolic extracts were analyzed by using aluminum backed silica gel plates of 250 μm layer, UV 254 (Whatman Ltd., Maidstone, Kent, England) with Co-chromatography. The plates were developed with a solvent system of toluene-methanol (80:20) with detection by spraying vanillin-sulfuric acid reagent (1.5g of vanillin and 0.5ml of concentrated sulfuric acid in 50 ml of Ethanol) and heating at 110°[11].

Phyton Inc., USA, carried out the HPLC analysis of extracts. The conditions of analysis were gradient method with pump A; water with 0.1% TFA and pump B; recovered acetonitrile 86% with TFA (0.1%) with a column of Water Deltapak C18,150x4.6mm, 4 μ with guard column. The flow rate of mobile phase was 0.85 ml/min.

The callus was pale yellowish in color and friable. It was easily dispersible into liquid media (0.5B5 with dicamba, 2 mg/l and Kn, 1 mg/l). The cell culture upon incubation at 25°, and at 120 rpm has grown with a growth index 2.2 in a week time. The cultures were maintained by sub culturing on weekly basis. If the culture were maintained by subculturing at two week interval, after 7 passages formed small aggregates in the flasks and leading non-producing cell culture. The 0.5 B5 medium (Dicamba+Kn) was found to be ideal for growth. The culture upon extraction for azadirachtin indicated negative.

The cultures were extracted and analyzed by TLC and HPLC. The TLC analysis of the cultures showed the same spots as the standard with R_f 0.56. The HPLC analysis showed the retention time of 10.13 minutes with standard. The UV absorption maxima was at 218 nm. The culture extracts which showed presence of azadirachtin had UV maxima, retention time and relative flow same as standard sample.

Of all the media tested for the production of azadirachtin in cultures, the medium no.1 produced maximum amount of azadirachtin upon analysis, out of 930 mg/l, 920 mg/l of azadirachtin was present in the cells, and the rest was in the medium. As the amount of azadirachtin was low, three flasks were pooled for extraction. The formation of aggre-

TABLE 2: EFFECT OF HORMONES (0.5 B5 MEDIUM WITH 3 % SUCROSE).

Hormones and their concentration	Azadirachtin (mg/l)
IAA (0.2 mg/l) + BA (0.2 mg/l)	450
2-4-D (2 mg/l) + Kn (0.5 mg/l)	300
NAA (1 mg/l) + Kn (0.5 mg/l)	476
Dicamba (2mg/l) + Kn (0.5 mg/l)	Not Detected

Kn represents kinetin, BA denotes benzyl adenine and NAA stands for napthaleneacetic acid

gates in medium no.5 may be due to zeatin. There are few reports indicating that zeatin induces somatic embryogenesis in plant cells[12]. Wewetzer[6] reported that for Nicaragua *A. indica* callus produced maximum amount of azadirachtin when grown on White's medium. However, in the case of *A. indica* A. Juss, no production of azadirachtin was observed. The reasons may be because of geographical location of the plant. Allan *et al.*[1] reported that the *A. indica* of Ghanaian strain callus cultures produced 0.0007% of dry weight basis of azadirachtin. There is only one report which identified the azadirachtin from the cell cultures of *A. indica* (Table 1).

Of all the hormonal combinations employed for the production of azadirachtin in *A. indica* cell cultures, MS medium with NAA (1 mg/l) and Kn (0.5 mg/l) produced the maximum amount of azadirachtin (476 mg/l). So the MS medium is best for the production while 0.5 B5 (Dicamba and Kn) for growth. In order to improve the yields of azadirachtin from cell cultures, the elicitation experiments were carried using production medium. Of all the elicitors tested, methyl jasmonate, MJ (1-500 mm), salacin (100-500 mm), cellulase (100 and 500 units), Sodium orthovandate (10-100 mm) do not have any effect on the production of azadirachtin.

In conclusion, 0.5 B5 medium with dicamba and Kn is suitable for growth and MS medium NAA and Kn for production. This is the first study to report 930 mg/l of azadirachtin from cell cultures of *A. indica*. However, before exploring the cell culture for commercial scale, the culture productivity has to be increased many folds.

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