Production of Azadirachtin in Anther Cultures of *Azadirachta indica* A. Juss., and Its Bioactivity Against *Aspergillus sydowii*

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Azadirachtin (78 μg/g dry weight) was detected in cell cultures established from anthers of *Azadirachta indica* A. Juss., a multipurpose tree belonging to the family Meliaceae. This is the first study which explicates the production of azadirachtin from *in vitro* anther cultures. Cell line maintained on Murashige and Skoog medium supplemented with kinetin (4.5 μM) and 2, 4-dichlorophenoxyacetic acid (0.5 μM) was selected based on its texture as well as rate of proliferation and utilized to test bioactivity against *Aspergillus sydowii*, a common fungus found to contaminate *in vitro* tissue cultures.

Keywords: Anther cultures, Antifungal activity, Azadirachtin, *Azadirachta indica*, Neem

Introduction

Azadirachta indica A. Juss. is a well-known medicinal tree of Indian subcontinent. Since time immemorial, different parts of the plant and their extracts have been employed to treat various diseased-body conditions all over the world. All the invaluable properties of neem are principally due to azadirachtin, a tetranortriterpenoid and a major bioactive constituent predominantly present in the seed kernels of this plant. Its presence in other parts like leaves is also reported, although in minor quantities. It possesses very potent biopesticidal and insect antifeedant properties. Apart from this, the compound has largely been found responsible for diverse type of bioactivities of neem, including antimicrobial properties.

Due to the strict cross breeding nature of the plant and resulting heterozygosity, a large variation has been found in the quantity of azadirachtin from plants growing in different areas of the world and even in the ones growing in the same locality. This makes the whole extraction procedure uneconomical. Chemical synthesis of this molecule is a herculean task because of its structural complexity. Recently, the chemical synthesis of the molecule was achieved by a group belonging to Cambridge University (Veitch *et al.*, 2007), 22 years after the discovery of this molecule.

Owing to all these limitations and industrial importance of azadirachtin, it is paramount to look into other options for the production of this compound. *In vitro* plant tissue culture offers yet another alternative for production of important secondary metabolites, sometimes

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even in quantities that allow economically feasible production (Wewetzer, 1998). This technique has several advantages over the whole plant material, as the whole procedure becomes less tedious; variability in the yield of metabolites due to seasonal fluctuations and conditions prevalent in the ecogeographical zones is prevented because the cultures are maintained in controlled conditions.

In the present study, we have evaluated *in vitro* anther cultures as a possible source for azadirachtin production. Apart from this, utility of these cultures has been proved in an antifungal assay on *Aspergillus sydowii*, a saprophytic fungus found in soil and a major cause of contamination in tissue cultures. Anther culture has advantages over other explants like seeds and leaves, as there are less chances of interference by other compounds hampering the purification of desired metabolite. Such cultures will be very useful for consistent production of chemicals. Efficacy of *in vitro* anther cell lines for azadirachtin production and antifungal activity was attempted for the first time through this study.

Materials and Methods

Plant Material and Initiation of Aseptic Cultures

Cultures were established during the flowering season of neem (April-May in India). Flowering twigs were collected in the morning between 7-8 a.m. Healthy flower buds were selected and surface sterilized with 0.1% mercuric chloride for 7 min, followed by three rinses in sterile distilled water. Thereafter, the buds were dissected and anthers were cultured on MS medium (Murashige and Skoog, 1962) containing macro and microsalts, vitamins, 100 mgL^{-1} myoinositol, enriched with 30 gL^{-1} sucrose and solidified with 0.8% agar (HiMedia Laboratories, India) with various combinations of auxins and cytokinins (Sigma, USA). The medium was adjusted to a pH of 5.8 before addition of agar and autoclaved at 121 °C, 15 psi for 15 min. 10 mL of medium was dispensed in each 60 mm petriplate (Tarsons, India). 20 anthers were cultured per plate and were incubated at 25 °C under 16/8 h light/dark regime, following an 8-week total dark exposure.

Maintenance of Fungal Cultures

Pure cultures of *A. sydowii* were obtained from University of Delhi, India. The cultures were maintained in Potato Dextrose Agar (PDA) medium that consisted of potato (200 g/L), dextrose (20 g/L), agar (17 g/L) and streptomycin (0.03 g/L) and adjusted to a pH of 7.3.

Preparation of Azadirachtin Standard

The stock solution of azadirachtin purchased from Sigma, Aldrich was prepared in methanol (HPLC grade, Merck, India) at a concentration of 1 mg/mL and stored at –20 °C. Calibration curve was generated by serially diluting the stock solution to five different concentrations. Each concentration was run at least thrice to check the repeatability of results.

Preparation of Plant Extract

The dried cell mass was soaked in methanol (analytical grade, Merck, India) overnight, followed by sonication for 35 min at 35% amplitude (pulser 5 s on/off cycle). The extract was centrifuged and filtered to remove the cell debris and further partitioned into aqueous

and dichloromethane (Merck, India) fractions. Dichloromethane extract was further analyzed for the presence of azadirachtin.

Chromatographic Conditions

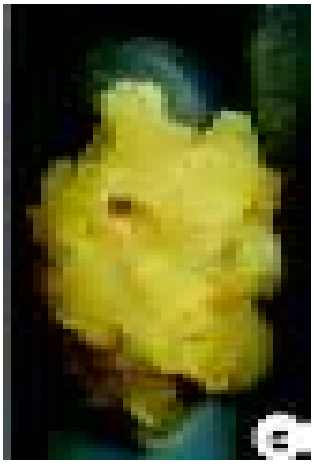
High Performance Liquid Chromatography (HPLC) was performed on a Varian Prostar HPLC system (Varian, USA) equipped with a prostar binary pump, UV detector and a 20 μL injection loop. Hypersil BDS RP-18 column (Thermo, USA) of dimensions 250 × 4.6 mm was used with methanol-water as the mobile phase at a flow rate of 0.5 mL/min and a detection wavelength of 214 nm.

Results and Discussion

Callus Induction

An explicit effect of growth regulators was witnessed right from the stage of callus induction and multiplication from anther culture of this tree species. Basal medium alone did not show any response at any stage of culture during the study. Also when either auxin(s) or cytokinin(s) were compounded with the medium, the response was poor during the initial stages. But the mixture of two auxins and one cytokinin proved to be beneficial for initial callus induction from the anthers, MS + 5 μM BAP + 1 μM 2, 4-D + 1 μM NAA served the purpose. On this combination, callusing started after four weeks of culture, and by eighth week, the anther wall pulled apart due to the pressure of callusing microspores releasing from inside the anther (Figure 1a). However, repeated subculturing on the same parental

Figure 1: Establishment of *In Vitro* Cell Lines from Anther Cultures

(a) 8-week-old Calli on Induction Medium, MS + BAP (5 μM) + 2, 4-D (1 μM) + NAA (1 μM)	(b) 15-week-old First Subculture on the Same Parental Combination	(c) 8-week-old Calli on MS + Kinetin (4.5 μM) + 2, 4-D (0.5 μM)
		
Note: Anther wall pulled apart longitudinally and releasing the callus from inside the anthers.	**Note:** Showing soggy and cream callus. Callus growth is very slow.	**Note:** This is the maintenance medium. The cream, friable, soft and moderate growth of callus is visible.

combination did not support good callus growth (Figure 1b). Instead, one auxin and one cytokinin combination was observed to be the best for improved callus multiplication and maintenance. Moderately growing, cream, friable and soft calli were established on MS + 4.5 μM Kn + 0.5 μM 2, 4-D (Figure 1c). This cell line was selected for the antifungal assay on the basis of its texture and rate of proliferation. Seven to eight weeks old subcultures were utilized for this study.

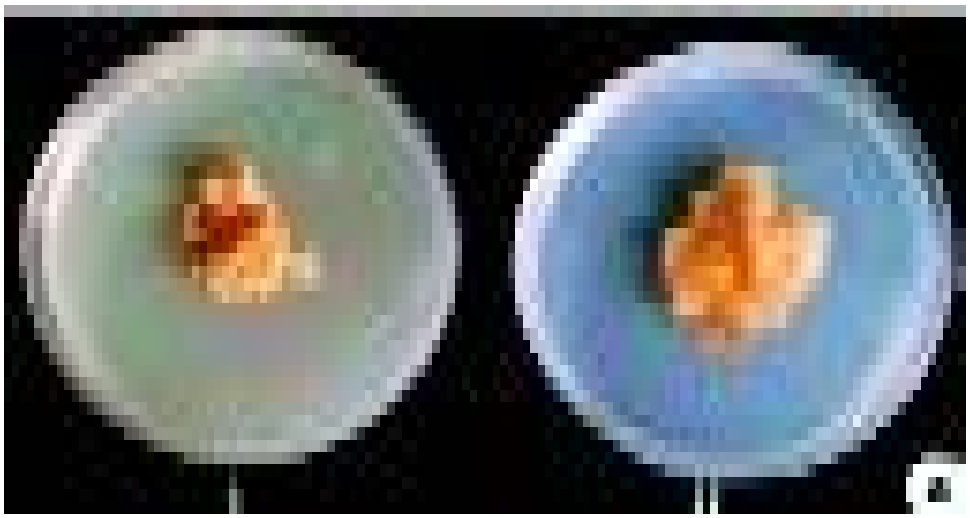
Antifungal Assay of Anther Callus

The inhibitory response of anther cell line maintained on MS + 2, 4-D (0.5 μM) + Kn (4.5 μM) against *A. sydowii* is shown in Figure 2. Two sets of controls were used for the experiments: the neem calli cultured on MS + 2, 4-D (0.5 μM) + Kn (4.5 μM) and on PDA medium served as control (Figure 2a) and the fungal cultures on these two media served as the second set of control (Figure 2b). As expected, the callus grew better on callus maintenance medium than on PDA medium. Similarly, *A. sydowii* grew on PDA medium and sporulated profusely within six weeks, but it remained largely vegetative on callus maintenance medium. To check the effect of callus on fungus, the callus was placed in the center of the plate and the fungal inocula were planted around it. The test fungus, *A. sydowii* showed inhibited growth when co-cultured with the neem anther callus on both the media (Figure 2c). In this study, a comparison of fungal growth in the presence and absence of callus clearly demonstrates the antifungal property of neem callus. Khan and Wassilew (1987) reported inhibitory effect of extracts of neem leaf, neem oil and seed kernels against certain human fungi including *Trichophyton, Epidermophyton, Mycosporum, Trichosporon, Geotricum* and *Candida*.

Figure 2: Demonstration of Antifungal Activity of Anther Cell Line Against *Aspergillus sydowii*

(a) i. 8 weeks old Callus Cultures on PDA Medium

ii. Same on MS + Kinetin (4.5 μM) + 2, 4-D (0.5 μM) Showing Healthy Calli

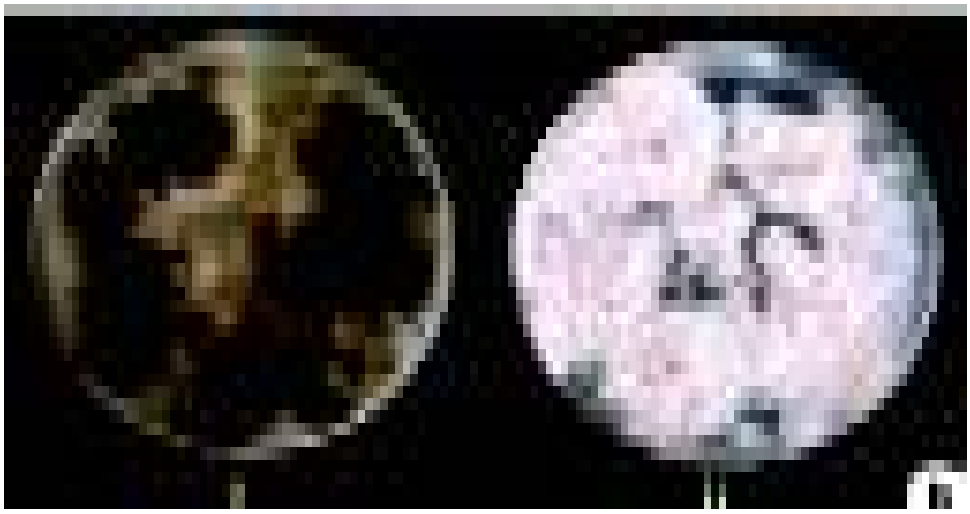


Control I

Figure 2 (Cont.)

(b) i. *A. sydowii* on PDA Medium Exhibiting Sporulation

ii. *A. sydowii* on Callus Maintenance Medium Remains Vegetative



Control II

(c) i. Calli Co-Cultivated with Fungus on PDA Medium

ii. Calli Co-Cultivated with Fungus on Callus Maintenance Medium (Inhibition of Fungal Growth Is Evident in Both (i and ii)

Co-Cultivation

Natarajan *et al*. (2002 and 2003) studied the effects of extract of leaves and seeds of *Azadirachta indica* on dermatophytes: *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum nanum*. They observed that the seed extract at concentration as low as

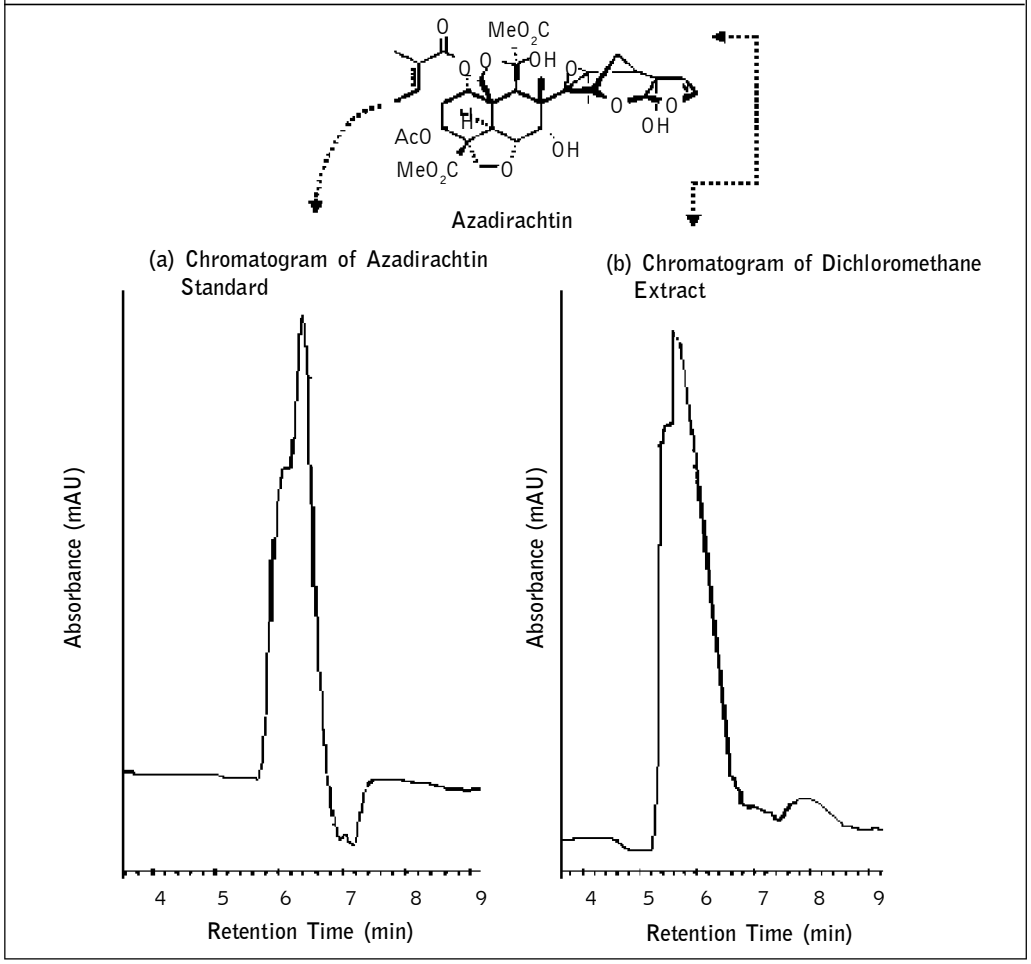
15 μg/mL was sufficient for distorting the growth pattern of the organisms tested. Mossini *et al.* (2004) observed inhibited patulin production when *Penicillium expansum* was cultured with aqueous extract of neem leaves.

The effects of the leaf aqueous extract from *Azadirachta indica* on the adhesion, cell surface hydrophobicity and biofilm formation of *Candida albicans* 12A and 156B were studied by Polaquini *et al.* (2006). These properties affect the colonization properties of the fungus. The researchers observed an anti-adhesive mechanism action in *Azadirachta indica* extract. However, till date no reports are available where source has been directly employed to check the antifungal activity.

Chemical Analysis

With the conditions described in materials and methods section, azadirachtin eluted at 6.39 min. HPLC analysis of dichloromethane extract of the callus line showing antifungal

Figure 3: HPLC of the Standard and Anther Cell Line Extract



activity revealed that azadirachtin constituted a major proportion of the extract with its amount reaching to 78 μg/g DW of callus (Figure 3). This amount of azadirachtin is higher than what was previously reported in callus cultures initiated from leaf and bark explants (Allan *et al.,* 1994; and Wewetzer 1998), flower explants (Veeresham *et al.*, 1998) and nodal segment explants (Raval *et al.,* 2003). Thus, anther cultures can also be an efficient alternative system for *in vitro* production of azadirachtin as found in the present study.

Conclusion

The present study proves the usefulness of anther cultures as a source of immensely important metabolite, azadirachtin. Moreover, the bioassay of anther-derived calli against *A. sydowii* further proves the utility of these cultures. ■

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