

seedlings and can save labour, space and cost. The RAPD analysis can also be applied to a broad array of cultivars and wild accessions to get a more accurate picture of the genetic diversity within the genus *Piper*.

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## Endophytic mycoflora of inner bark of *Azadirachta indica* A. Juss

Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate overt negative effects<sup>1</sup>. They are a largely unexplored component of biodiversity, especially in the tropics. Endophytic fungi have been isolated from leaves, stems and roots of woody plants in the temperate regions and the tropics<sup>2–4</sup>. They have a protective role against insect herbivory and many are potential producers of novel antimicrobial secondary metabolites<sup>5</sup>. Endophytes are constantly exposed to intergeneric–genetic exchange with the host plant. Isolation of a potent anticancer agent, taxol from *Pestalotiopsis microspora*, an endophyte of the yew tree and the phytohormone-producing fungus from rice plant, *Gibberella fujikuroi* suggests the potential of endophytes as a source of useful metabolites<sup>6,7</sup>.

The current study was carried out to isolate and identify fungal endophytes from living symptomless inner bark tissues of neem (*Azadirachta indica* A. Juss), which is an indigenous medicinal plant in India and Africa. Neem is an evergreen tree of the tropics and sub-tropics belonging to the family Meliaceae. It is widely used in Indian traditional medicine for various therapeutic purposes as well as the source of agrochemicals for many centuries. The bark extract has been scientifically investigated from the past two decades for anti-bacterial, antipyretic<sup>8,9</sup>, anti-inflammatory<sup>10</sup> effects

and against skin diseases such as eczema, burns, ulcers, herpes, etc.<sup>11</sup>. Based on the recent claims that endophytic microbes may play a key role in the therapeutic properties of plants, we postulate that the healing properties may be due to the secretion of metabolites from the endophytes residing in the bark.

Bark samples from a neem tree growing in Mysore were obtained by cutting the tree bark at 1.5 m above the ground level and 1–1.5 cm depth with ethanol-disinfected machete. Approximately 5 × 5 cm bark pieces were taken for the study. The samples were processed within 24 h of collection. Surface sterilization of bark sample was done by immersing the bark pieces in 70% (v/v) ethyl alcohol for 1 min and 3.5% (v/v) sodium hypochlorite for 2 min and rinsed three times in sterile distilled water for 1 min<sup>12</sup>. Excess water was blotted in an airflow chamber. The outer bark was removed and the inner portion containing the cortex was carefully dissected into bits (1.0 × 0.2 cm). 200 segments were plated on water agar medium (15 g l<sup>-1</sup>) amended with streptomycin (100 mg l<sup>-1</sup>) and incubated in a chamber for 21 days at 12 h light/dark cycles at 22°C<sup>13</sup>. The plates were monitored regularly for the growth of endophytic fungi. The hyphal tips that grew on surface-sterilized bark pieces were isolated onto potato dextrose agar (PDA). Each fungus was assigned a number and stored

at 4°C. Endophytic fungal strains were identified based on morphological characters using standard identification manuals. All the endophytic isolates were documented, maintained in cryovials on PDA layered with 15% glycerol (v/v) and stored in –80°C freezer (Cryo Scientific Pvt Ltd, Chennai) at the Department of Applied Botany and Biotechnology, University of Mysore.

The per cent frequency of occurrence<sup>14</sup> was calculated as the number of bark segments colonized by a specific fungus divided by total number of segments plated × 100 and dominant endophytes<sup>15</sup> were calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes × 100.

A total of 77 endophytic fungal isolates belonging to 15 genera were isolated from the inner bark of *A. indica*. The colonization frequency was 38.5% (Table 1). The fungal composition included 71.4% of hyphomycetes, 18.2% of coelomycetes, 6.5% of ascomycetes and 3.9% of sterile mycelia.

In the tropics, only a few studies have been carried out on endophytes of tree species<sup>16</sup>. Rajagopal and Suryanarayanan<sup>17</sup> have investigated the endophytic fungi in the leaves of *A. indica*. These studies have shown the effect of leaf tissue type, site and seasonality on endophyte assemblages and colonization. They recorded only *Fusarium* spp. and some sterile fungi. We have recovered endophytic genera like

**Table 1.** Endophytic fungi isolated from inner bark of neem (*Azadirachta indica*)

Endophytic fungi	No. of endophytes	Colonization frequency*	Dominant fungi
<b>Ascomycetes</b>			
<i>Chaetomium crispatum</i>	1	0.5	1.3
<i>Chaetomium globosum</i>	4	2.0	5.1
<b>Coelomycetes</b>			
<i>Pestalotiopsis</i> spp.	12	6.0	15.5
<i>Phoma eupyrena</i>	1	0.5	1.3
<i>Phyllosticta</i> spp.	1	0.5	1.3
<b>Hyphomycetes</b>			
<i>Acremonium acremonium</i>	1	0.5	1.3
<i>Aspergillus flavus</i>	4	2.0	5.1
<i>Aspergillus niger</i>	5	2.5	6.4
<i>Aspergillus oryzae</i>	1	0.5	1.3
<i>Cladosporium acaciicola</i>	1	0.5	1.3
<i>Cladosporium cladosporioides</i>	3	1.5	3.9
<i>Cochlonema verrucosum</i>	1	0.5	1.3
<i>Curvularia lunata</i>	1	0.5	1.3
<i>Fusarium clamydosporum</i>	1	0.5	1.3
<i>Fusarium moniliformae</i> var. <i>subglutinans</i>	2	1.0	2.6
<i>Fusarium oxysporum</i>	1	0.5	1.3
<i>Fusarium solani</i>	2	1.0	2.6
<i>Gliomastix</i> spp.	1	0.5	1.3
<i>Nigrospora oryzae</i>	2	1.0	2.6
<i>Penicillium</i> spp.	9	4.5	11.6
<i>Trichoderma</i> spp.	18	9.0	23.3
<i>Verticillium albo-atrum</i>	2	1.0	2.6
Sterile mycelia	3	1.5	3.9
No. of isolates	77	38.5%	

\*Based on the 200 segments plated.

*Curvularia*, *Cochlonema*, *Gliomastix* and *Verticillium* spp., which are reported as endophytes. *Trichoderma*, *Penicillium* and *Pestalotiopsis* spp. were the most dominant endophytes isolated in this study. Endophytic genera such as *Phomopsis*, *Phyllosticta* and *Xylaria* are commonly isolated from tropical and temperate regions<sup>12</sup>. Some species of *Fusarium* are pathogenic to crops, since some phytopathogenic fungus can be modified by mutation to grow as a non-pathogenic endophyte<sup>18</sup>.

Suresh *et al.*<sup>19</sup> reported the presence of limonoids in the leaf of neem as antifungal and perhaps this is the reason for a low score of endophytes, as reported by Rajagopal and Suryanarayanan<sup>17</sup>. The occurrence of endophytes seems to be influenced by seasonal variation<sup>20</sup>. The occurrence of fungal endophytes is mainly influenced by environment and type of host tissue<sup>2</sup>. Fungal species like *Trichoderma* are reported to have growth-promoting activity when cultivated with rice seedlings<sup>21</sup>. *Penicillium* spp. have been found to produce important antibiotics, which weaken or kill bacteria and other organisms that can cause disease. *Pestalotiopsis* spp. obtained

as endophytes in the Himalayan yew (*Taxus wallichiana*) produce taxol, an important chemotherapeutic drug used in the treatment of breast and ovarian cancers<sup>22</sup>. We are currently pursuing fermentation of these microbes to obtain the secondary metabolites to facilitate screening against therapeutic targets as well as against economically important plant pathogens.

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## Efficacy of *Hyptis suaveolens* against lepidopteran pests

*Hyptis suaveolens* (L.) Poit, a rigid sweetly aromatic herb belonging to the family Lamiaceae is a native of tropical America. It was introduced and naturalized in India<sup>1</sup>. The plant is used as green manure in certain parts of the west coast. The edible shoot tips are sometimes used for flavouring. In Java, the plant is used as cattle fodder. An infusion of the plant is used to treat catarhal conditions, affections of the uterus and parasitical cutaneous diseases; the leaf juice is taken internally for colic and stomach-aches. The Mundas (group of tribals from Orissa and West Bengal) use the plant for headache; the powder of leaf is used as snuff to stop bleeding of the nose. In Philippines, the leaves are used for anti-spasmodic, antirheumatic and antisporific baths. A decoction of the roots is used as appetizer and the root is chewed with betel nuts as a stomachic<sup>2,3</sup>. The leaves are used to treat cancer<sup>4</sup> and anti-fertility<sup>5</sup>.

Some species of *Hyptis* have been shown to possess insecticidal properties. Insecticidal activity of volatile oils from *Hyptis martiusii* has been reported<sup>6</sup>. Also the chemical compositions of the essential oil from *H. martiusii*<sup>6</sup>, *H. mutabilis*<sup>7</sup>, *H. suaveolens*<sup>8-10</sup>, *H. spicigera*<sup>11</sup>, *H. verticillata*<sup>12</sup>, *H. crenata*<sup>13</sup> and *H. pectinata*<sup>14</sup> have been reported.

The presence of ethereal oil, monoterpenes, diterpenes, suaveolic acid, suaveolol, triterpenoid, traces of hydrocyanic acid, sterol, campesterol, fucosterol, sesquiterpene alcohols and fatty acids<sup>1,9,10</sup> have been reported in this plant. 1,8-cineole and sabinene are the main constituents<sup>10</sup>.

In India, *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae) is a serious pest feeding on more than 180 host plants belonging to 45 families<sup>15</sup>. It commonly destroys more than half the yield. The annual loss amounts to US\$ 300–500 million in cotton and pulses<sup>16</sup>. *Spodoptera litura* Fab. (Lepidoptera: Noctuidae) is another economically important insect pest of cosmopolitan distribution<sup>17</sup>. It has been reported to attack more than 112 different species of cultivated crop plants throughout the world<sup>18</sup>. Both the noctuids feed on tender leaves, flowers and immature pods and ultimately cause severe loss of production. Growing awareness on the negative impact of chemical pesticides on the environment has prompted a surge to look for alternatives. Plants have been identified to play a vital role in providing

alternative source of biodegradable pesticides. The present study was undertaken to identify some new chemical compounds from *H. suaveolens* to control lepidopteran pests.

Fresh mature leaves of *H. suaveolens* were collected from Chennai, shade-dried and powdered using electric blender. One kg of plant powder was soaked in each solvent (hexane, diethyl ether, dichloromethane, ethyl acetate, methanol and water) for 24 h at room temperature ( $28 \pm 2^\circ\text{C}$ ) sequentially and filtered. The solvent from the crude was evaporated using rotary vacuum evaporator, weighed and stored at  $4^\circ\text{C}$  for subsequent experiments. From the crude, 1000 ppm concentration was prepared and tested for antifeedant, oviposition deterrent, ovicidal and larvicidal activity against lepidopteran pests, *Helicoverpa armigera* and *Spodoptera litura*.

Antifeedant activity of the plant extracts was studied using leaf disc no choice method<sup>19</sup>. Fresh leaf discs (3-cm diameter) of castor and cotton were used for *S. litura* and *H. armigera* respectively. The leaf discs were treated with 1000 ppm concentration of plant extracts individually; one treatment with acetone alone was used as positive control and one treatment without solvent was considered as negative control. In each petri dish (1.5 cm  $\times$  9 cm) wet filter paper was placed to avoid early drying of the leaf disc and single fourth instar larva of *S. litura* and *H. armigera* was introduced individually. Five replicates were maintained for each concentration and the progressive consumption of leaf area by the larva after 24 h was recorded in control and treated discs using leaf area meter (Delta-T Devices, Serial No. 15736 F 96, UK).

For oviposition deterrent activity 1000 ppm concentration of plant extracts was sprayed on fresh castor and cotton leaves for *S. litura* and *H. armigera* respectively; similar controls as mentioned above were also used here. The petioles of the treated leaves were tied with wet cotton plug to avoid early drying and placed inside the cage (60 cm  $\times$  45 cm  $\times$  45 cm). Ten pairs of *S. litura* and *H. armigera* moths were introduced on castor and cotton leaves respectively. 10% (w/v) sucrose solution with multivitamin drops was provided for adult feeding to increase fecundity. Five replicates were maintained for control and treatments. After 48 h, the numbers of egg masses

(*S. litura*) and eggs (*H. armigera*) laid on treated and control leaves were recorded and the percentage of oviposition deterrence was calculated<sup>20</sup>.

For ovicidal activity, scales from the egg masses of *S. litura* were carefully removed using fine camel brush. 500 eggs from both the lepidopterans were separated into 5 lots each having 100 eggs and dipped in 1000 ppm concentration of plant extracts and controls as mentioned above. Number of eggs hatched in control and treatments were recorded and the percentage of ovicidal activity was calculated using Abbott's formula<sup>21</sup>.

For evaluation of larvicidal activity against *S. litura*, fresh castor leaves were treated with 1000 ppm concentration of plant extracts and controls as mentioned above. Petioles of the leaves were tied with wet cotton plug to avoid early drying and placed in plastic trough (29 cm  $\times$  8 cm); 20 starved (2 h) IV instar larvae of *S. litura* were introduced individually and covered with muslin cloth. For *H. armigera* 1000 ppm concentration of plant extracts was mixed with artificial diet<sup>22</sup>. Small pieces of artificial diet were separated and placed in plastic containers. Single IV instar larva was introduced in each container. Five individual containers were considered as one replication. Five replicates were maintained and the number of larvae dead after 48 h was recorded and the percentage of larval mortality was calculated using Abbott's formula<sup>21</sup>. All the data collected were subjected to Analysis of Variance (ANOVA) and the significant difference within the mean was separated using Least Significant Difference test (LSD;  $P < 0.05$ ).

Crude ethyl acetate extract (20 g) was dissolved in 10 ml of ethyl acetate and 5 g of silica gel and macerated well using mortar and pestle to make fine powder. The powdered material was fractionated through a silica gel (100–200 mesh LR) column chromatography (4 cm  $\times$  60 cm) using the combination of hexane/ethyl acetate (95 : 5; 90 : 10; 85 : 15; 80 : 20). Totally 11 fractions were obtained; each fraction was confirmed using Thin Layer Chromatography (on Aluchrosep Silica gel 60 UV<sub>254</sub> gel coated sheets); each fraction was tested for its bioactivity at 500 ppm concentration. Promising fractions were further studied for their bioactivity at 100, 250, 500, 1000 and 2000 ppm. Purified promising fractions were subjected