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European Journal of Experimental Biology, 2011, 1 (2):219-225

Investigation of phylloplane mycoflora from some medicinal plants

***[1]Prabakaran, M., [2]Merinal, S. and [3]Panneerselvam, A**

[1] Sri Gowri Biotech Research Academy, No. 5, Gowtham Nagar, Thanjavur
[2.] Department of Botany, Kunthavai Naacchiyaar Govt. Arts College for Women (autonomous), Thanjavur
[3] PG and Research Department of Botany and Microbiology, A.V.V.M Sri Pushpam College, Poondi, Thanjavur

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ABSTRACT

Survey on the occurrence of phylloplane fungi on leaves surface of three important medicinal plants such as Ocimum sanctum, Phyllanthus amarus and Azadirachta indica. A total number of 10 fungal species belonging to five genera were isolated from surface sterilized leaf segments of by dilution plating technique. Among them Aspergillus flavus, Penicillium expansum, Fusarium semitectum, Fusarium oxysporum were isolated from the phylloplane of Ocimum sanctum. Scopulariopsis sp. was isolated from the phylloplane of Phyllanthus amarus. Penicillium janthinellum, Aspergillus fumiculosis, Aspergillus sp., Curvularia lunata and Fusarium moniliforme were isolated from the phylloplane of Azadirachta indica. The phytochemical screening of the medicinal plant revealed that the flavonoids, cardiac glycosides, terpenoids were found in Ocimum sanctum and Azadirachta indica and not found in Phyllanthus amarus.

Key words: phylloplane, medicinal plants, dilution plating technique, phytochemical.

INTRODUCTION

Biodiversity is the variation of life forms within a given ecosystem. Biodiversity is often used as a measure of the health of biological systems. Biodiversity of fungi is essential for anyone collecting or monitoring any fungi. Fascinating and beautiful fungi are vital components of nearly all ecosystems and impact human health and our economy in a myriad of ways. Standardized methods for documenting diversity and distribution have been lacking. A wealth of information, especially regarding sampling protocols, compiled by an international team of fungal biologists, make biodiversity of fungi an incredible and fundamental resource for the study of organismal biodiversity.

A fungus is a member of a large group of eukaryotic organism that includes microorganisms such as yeast and molds. Fungi are classified as a kingdom that is separate from plants, animals and bacteria. One major difference is that fungal cells have cell walls that contain chitin, unlike the cell walls of plants, which contain cellulose.

The phylloplane, the surface of plant leaves is a complex terrestrial habitat that is characterized by a variety of microorganisms including bacteria, filamentous fungi and yeast. Phylloplane fungi are the mycota growing or the surface of leaves. There are two groups of phylloplane fungi: residents and casuals. Residents can multiply on the surface of healthy leaves without noticeably affecting the host. Whereas, casuals land on the leaf surface but cannot grow. Phylloplane fungi have been poorly studied as compared to endophytes, saprobes and pathogenic fungi.

Medicinal plants have been of age long remedies for human diseases because they contain components of therapeutic value [13]. Different parts of *Alpinia galanga* are traditionally claimed to be used for the treatment of ailments including anti-fungal, anti-tumor, Antihelmintic, anti-diuretic, anti-ulcerative, disease of heart, rheumatic pains, chest pain, dyspepsia, fever, diabetes, burning of liver and kidney disease [19]. Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flowers leaves and roots that work with nutrients and fibers to act defense system against disease or more accurately, to protect against disease. Phytochemicals are divided into two groups, which are primary and secondary constituents, according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acids, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoids, phenolic compounds [9] and many more such as flavonoids, tannins and so on.

- *Azadirachta indica*
- *Phyllanthus amarus* and
- *Ocimum sanctum*

The above plants are chosen to study because they come in abundant source, easily available and some of them are already being utilized in traditional medicine. By studying the presence of phytochemical in these plants, the uses of these plants in traditional treatment can be explained scientifically.

MATERIALS AND METHODS

Sample Collection

The fresh leaves of *Ocimum sanctum, Phyllanthus amarus* and *Azadirachta indica* were collected from the surrounding area of Thanjavur and immediately brought to the laboratory.

Media Preparation

Composition of Potato Dextrose Agar Medium

Potato (peeled)	-	250gm
Dextrose	-	15 gm
Agar	-	18 gm
Distilled water	-	1000 ml

Preparation of Potato Dextrose Agar Medium

The potato tubers were peeled and weighed for about 250g. The tubers were chopped into small pieces with the help of sterile knife. The chopped potatoes were transferred into a conical flask containing about 1000ml of distilled water. The content was boiled for 20 min. The supernatant were decanted and filtered by muslin cloth and the filtrate was collected. Dextrose (15g) and agar (18g) were transferred into the extract and shaked to dissolve the ingredients.

The medium was made up to 1 litre by addition of distilled water. The pH of the medium was adjusted to 5.6. Finally, the medium was cotton plugged and autoclaved at 121ºC for 15 minutes.

Isolation of fungi

Leaf samples of *Ocimum sanctum, Phyllanthus amarus* and *Azadirachta indica* were collected and placed in sterile plastic bags, and immediately brought to the laboratory. From the basal part of the leave a fragment of 1cm of leaf blade was cutout and shaken in flasks filled with 200ml of distilled water. From the suspension of microorganisms prepared in this way 0.2ml was transferred into petridishes containing potato dextrose agar medium with streptomycin. The inoculum was spreaded uniformly and kept undisturbed in dust free chamber at room temperature for a period of 3-5 days. The fungal colonies were observed and pure cultures were maintained.

Lacto Phenol Cotton Blue Mounting

A portion of the mycelium of the representative colonies was picked up with the help of a pair of needles and semi permanent slides were prepared using lactophenol cotton blue (20g – phenol (crystal); 20g lactic acid; 40g glycerin; 20 ml water; cotton blue a few drops). The slide was gently heated in a sprit lamp so as to release the air bubbles, if any present inside the cover glass. The excess stain was removed using tissue paper and the cover glass was sealed using white nail polish.

Identification and photomicrography

As the plating method yield facultative phylloplane fungi were identified referring the standard manuals. The manual of soil fungi [6], and Dematiaceous Hyphomycetes and More Dematiaceous Hyphomycetes [4]. The photographs were taken using Nikon microscope.

Phytochemical Analysis

Processing of plant samples

The leaves of the plants are properly washed in tap water and then rinsed in distilled water. The rinsed leaves are dried in an oven at a temperature of 35 – 40ºC for 3 days. The dried leaves of each plant are pulverized, using a sterile electric blender, to obtain a powdered form. The powdered form of these plants is stored in airtight grass containers, protected from sunlight until required for analysis.

Preliminary Phytochemical Screening of Medicinal plants

Phytochemical tests were carried out on the aqueous and ethanol extract and on the powdered specimens using standard procedures to identify the constituents as described by [16, 17 & 7].

Qualitative analysis

Test for steroids

Two ml of acetic anhydride was added to 0.5g ethanolic extract of each sample with 2ml sulphuric acid. The colour change from violet to blue or green indicating the presence of steroids.

Test for Terpenoids

Five ml of each extract was mixed in 2ml of chloroform, and concentrated sulphuric acid (3ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed indicating the presence of terpenoids.

Test for Cardiac Glycosides (Keller Killani test)

Five ml of each extracts was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was under layed with 1ml of concentrated sulphuric acid. A brown ring of the interface was formed indicating the presence of cardiac glycosides.

Test for flavonoids

Five ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated sulphuric acid. A yellow colouration was observed indicating the presence of flavonoids.

Test for saponins

About 2g of the powdered sample was boiled in 20ml of distilled water in a water bath and filtered. 10ml of the filtered sample is mixed with 5ml of distilled water in a test tube and shaken vigorously to obtain a stable persistent froth. The frothing is then mixed with 3 drops of olive oil. Formation of emulsion indicating the presence of saponins.

RESULTS

Biodiversity

A total number of 10 fungal species were isolated from surface sterilized leaf segments of three important medicinal plants such as *Ocimum sanctum*, *Phyllanthus amarus* and *Azadirachta indica* by dilution plating technique. Among them *Aspergillus flavus, Penicillium expansum, Fusarium semitectum, Fusarium oxysporum* were isolated from the phylloplane *of Ocimum sanctum. Scopulariopsis* sp. was isolated from the phylloplane of *Phyllanthus amarus. Penicillium janthinellum, Aspergillus fumiculosis, Aspergillus* sp., *Curvularia lunata* and *Fusarium moniliforme* were isolated from the phylloplane of *Azadirachta indica.*

Phytochemical Analysis

The present study was carried out in the plant samples revealed the presence (or) absence of medicinally active constituents. Saponins were present in *Phyllanthus amarus* and *Azadirachta indica*. Steroids were absent in all the three medicinal plants. But the flavonoids, cardiac glycosides, terpenoids were found in *Ocimum sanctum* and *Azadirachta indica* and not found in *Phyllanthus amarus* (Table-2).

Table 1: Fungi isolated from the Phylloplane of medicinal plants

S. No.	Medicinal plants	Fungal species
1.	*Ocimum sanctum*	(i) *Aspergillus flavus* (ii) *Penicillium expansum* (iii) *Fusarium semitectum* (iv) *Fusarium oxysporum*
2.	*Phyllanthus amarus*	(v) *Scopulariopsis* sp.
3.	*Azadirachta indica*	*(vi)* *Penicillium janthinellum* *(vii)* *Aspergillus fumiculosis* *(viii)* *Aspergillus* sp. *(ix)* *Curvularia lunata* *(x)* *Fusarium moniliforme*

Table- 2: Preliminary phytochemical screening of *Ocimum sanctum, Phyllanthus amarus* and *Azadirachta indica*

Phytochemical constituents	*Ocimum sanctum*	*Phyllanthus amarus*	*Azadirachta indica*
Steroids	-	-	-
Terpenoids	+	-	+
Cardiac glycosides	+	-	+
Flavonoids	+	-	+
Saponins	-	+	+

(+) = *Positive*
(-) = *Negative*

DISCUSSION

The variety and galaxy of fungi and their natural beauty occupy prime place in the biological world. One third of fungal diversity of the globe exists in India. Out of 1.5 million of fungi, only 50% are characterized till now. Unfortunately, only around 5 – 10% of fungi can be cultured artificially. Fungi play a significant role in the daily life of human beings besides their utilization in industry, agriculture, medicine, food industry, textiles, bio remediation and many other ways fungal biodiversity has become an integral part of the human welfare [11]. Phylloplane communities may be similar on different plants such as mangrove plants and terrestrial and non marine plants [10].

In the present study totally 10 species of fungi were isolated from the phylloplane of medicinal plants by dilution plating technique. They were *Aspergillus flavus, Aspergillus fumiculosis, Aspergillus* sp., *Penicillium expansum, Penicillium janthinellum, Fusarium semitectum, Fusarium moniliforme, Fusarium oxysporum, Curvularia lunata* and *Scopulriopsis* species. [3] reported that survey on the occurrence of phyllosphere and phylloplane fungi on leaves surface of 15 different kinds of fresh herbs. Seventy seven species belonging to 37 genera of fungi were isolated and identified. *Alternaria, Aspergillus* and *Cladosporium* were the most common genera.

Although the present study deals with the diversity of fungal communities associated with some medicinal herbs, the actual diversity may depend on the methods used for gathering and handling leaf samples, size of the leaf fragments and culture.

A total of 2159 isolates belonging to 55 different fungal species were recovered from 3600 leaf segments incubated from 9 medicinal herbs. The frequency of fungal species differed significantly between the wet and dry season. The number of fungi and frequency of colonization were greater during the wet season than the dry season. This may be because the greater rainfall in the wet season could promote the dispersal of fungal spores [2].

The number of fungal colonies and the percentage cover of phylloplane fungi can be estimated by direct observation using light microscopy [18]. The leaf washing method cannot provide a quantitative description of the species richness of the phylloplane fungi as leaf washings only indicate the amount of porpagules, but not the fungal biomass. Thus the leaf washing method is suitable for qualitative studies only [12].

Phytochemical Screening

In the present investigation the phytochemical screening of the medicinal plant studies showed that the leaves were rich in flavonoids, terpenoids, cardiac glycosides and saponins. They were known to show medicinal activity as well as exhibiting physiological activity. The presence of saponins in the leaves of *Azadirachta indica* explains why the leaves of *Azadirachta indica* used for hypertension treatment. The presence of tannins also aids in wound healing[14]. *Azadirachta indica* leaves are used to treat chicken pox by directly applying to the skin in a paste form. From the phytochemical analysis, it is known that *A. indica* is a source of terpenoid, which play an important role in wound and scar healing [8]. Anthraquinone compounds are present in *Cassia tora* Linn [5].

Several medicinal properties have been attributed to *Ocimum sanctum.* The leaves of *Ocimum sanctum* are said to have abortifacient effect in women. Essential oils extracted from the leaves of *O. sanctum* has been found to inhibit *invitro* growth of *Bacillus anthracis, Escherichia coli,* and *Pseudomonas aeruginosa* showing its antibacterial activity [1]. The juice of fresh leaves of *O. sanctum* also given to patients to treat chronic fever, dysentery, hemorrhage and dyspepsia [15].

The leaves of *Phyllanthus amarus* has been employed in Ayurvedha for the treatment of Jaundice, Cough and labored breathing. It also migrates disorders of blood and bleeding. This plant is also considered to be gastric stimulant, carminative, antidiabetic, antimalarial, anti inflammatory, analgesis, antiseptic, detoxicant and efficacious in dropsy, splenic disorders and skin diseases. Thus the phytochemical screening on qualitative analysis shows that the leaves of *Azadirachta indica* and *Ocimum sanctum* are rich in flavonoids, saponins, terpenoids and cardiac glycosides. The steroid is absent in both these plants. But the leaves of *Phyllanthus amarus* only rich in saponins.

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