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Enumeration of phylloplane and endophytic fungi from medicinal plant, *Solanum nigrum* by two different techniques

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Abstract: Isolation and enumeration of endophytic and phylloplane fungi from different leaf samples of the medicinal plant, Solanum nigrum was carried out in the Microbiology laboratory, Department of Botany, K.M. Center for P.G Studies (Autonomous), Pondicherry during early 2015. Agar plate and moist chamber techniques were used to isolate the endophytic and ectophytic fungi. During the study period, a total of 16 fungi were isolated under 10 genera from both agar plate and moist chamber plates. Incidence of the fungi isolated by moist chamber was less than agar plates. In agar plate technique, *Penicillium digitatum, Aspergillus awamori, Alternaria* sp., recorded from the leaf samples but, in moist chamber, *Torula herbarum, Trichoderma harzianum* and *Aspergillus flavus* were recorded from the leaf samples. *Alternaria* sp., *Cladosporium herbarum, Aspergillus awamori, A. flavipes, A. niger Monascus sp., Penicillium citrinum, P. digitatum, Torula herbarum* and *Trichoderma harzianum* were identified as phylloplane fungi and *Alternaria* sp., *Aspergillus niger, Aspergillus flavus*, Brown sterile mycelia, *Penicillium chrysogenum, P. digitatum, Torula herbarum, Trichoderma* sp. and White sterile mycelia were recognized as endophytes. Of all fungi recorded, *Torula herbarum* was found as the true endophytes among the total number of isolated phylloplane and endophytic fungi from the leaf samples of *Solanum nigrum* by two different methods. Moist chamber method was found to be easy to isolate endophytic and phylloplane fungi correctly in comparison to agar plate method, where the sporulation of fungi was very fast than the moist chamber.
Key words: Phylloplane, Endophytic fungi, Medicinal plant, *Solanum nigrum.*

Introduction

Fungi lives in the endophytic region of the leaves are a good source of antibiotics. Such endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites. The varied relationships exist between fungal endophytes and their host plants ranging from mutualistics to antagonistic or slightly pathogenic[1,2]. Endophytic fungi are many orders of fungi with colonize of living, internal tissue of plants without any immediate or overt negative effects[3]. These estimates do not include additional sources of fungal diversity[4]. In other way, phylloplane fungi are the inhabitants of leaf surfaces and promote their growth and sporulation by the virtue of varied nutrients available in the leaf [5]. Endophytic fungi represent an important and quantifiable component of fungal biodiversity and structure[6,7]. Endophytic fungi are unexplored group of organisms that has enormous potentials for new pharmaceutical substances. There have been several studies on the phylloplane fungi of mangroves in Tamilnadu state[8]. Most phylloplane fungal studies have been concentrated with pathogens or non-parasitic fungi of crops or economically-important trees[9]. Puducherry district holds a very different type of vegetation where, the isolation and identification of

endophytic and phylloplane fungi from medicinal plants their antibacterial and antifungal activities are lacking. The present study on isolation and enumeration of endophytic and phylloplane fungi from one medicinal plant i.e., *Solanum nigrum* by different techniques were carried out in the Microbiology laboratory, Department of Botany, K. M. Centre for P. G. Studies (Autonomous) campus, Pondicherry during first half of 2015.

Materials And Methods

Sample collection

Leaves of *Solanum nigrum* were collected in fresh condition from Tagore Arts College campus, Lawspet, Puducherry- 605008. Healthy and mature leaf samples were carefully isolated and brought to the Microbiology Laboratory, Department of Botany with utmost care and kept in room temperature for further experiments.

Description of the Plant

Botanical name : *Solanum nigrum*
Family : Solanaceae
Common name : Black night shade
Vernacular name : Manathakkali

The plant is known as black night shade common herb or short – lived perennial shrub, found in many wooded areas, as well as disturbed habitats. The plant looks erect, angular, branching and the stem grows up to 1 to 2 feet high and may be glabrous or covered with inward – bent hairs. The leaves are mostly alternate, dark green, ovate and wavy toothed or nearly entire. The fruits are many –seeded, pea –sized, purple or black berry types.

Medicinal uses

There are a number of medicinal values of this plant, if it is taken internally in very small amounts, the leaves strongly promote perspiration and purge the bowels on the next day. The fresh juices of the herb are sometimes used for fever and allay pain. Its large doses, black night shade can cause serious, but usually not fatal poisoning. Externally, the juice or an ointment prepared from the leaves can be used for the skin problems and tumors. The berries are poisonous, but boiling apparently destroys the toxic substances and makes them usable for preserves of jams and pies. The fruits are used as cosmetics; rubbing the seeds on the checks to remove freckles, children harmlessly and extensively eat the mature fruit. The fruit also used for diabetes. Decoction of stalk leaves and roots are good for wounds and cancerous sores. An infusion of the plant is used as an enema in infants having abdominal upsets. Freshly prepared plant extracts is effective in the treatment of cirrhosis of the liver and also serves as an antidote to opium poisoning.

Surface sterilization of leaves

In order to isolate the endophytic fungi, the collected healthy leaves were thoroughly washed in running tap water. Then the leaves were cut into small segments (about $1cm^2$) including midrib portion. The leaf samples were surface sterilized by 0.1 % mercuric chloride for 60 seconds and then rinsed in sterile distilled water for 10 seconds (three times). For phylloplane mycoflora study, the leaf segments were not surface sterilized since phylloplane fungi grown on the surface of the leaves. Without washing the segments, they were placed on the PDA and moist chamber plates equidistantly.

Culture of leaf samples on agar plates

Leaf segments of a centimeter square, both sterile an unsterile were placed separately on the PDA media plates equidistantly by the help of sterile forceps and pressed later on followed by incubation for 3 to 7 days.

Culture of leaf sample on moist chamber

Same like agar plates, leaf segments of centimeter square, both sterile an unsterile were placed separately on the moist chamber petriplates equidistantly by the help of sterile forceps and pressed later on followed by incubation for 7 to 21 days. The fungi on moist chamber were enumerated later on based on their growth on the leaf segments.

Isolation of fungi

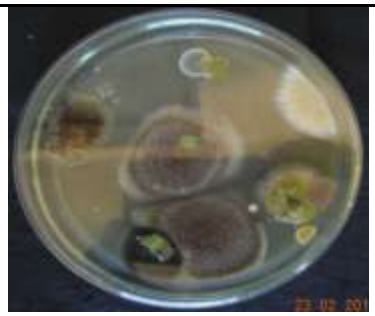
After sterilization, the excess water was blotted out by sterile filter paper from the leaf segments and kept separately. Then the surface sterilized segments were placed in a petridishes containing PDA supplemented with Tetracycline as well as in moist chamber. The moist chamber plates don't need any type of medium for the growth of endophytic as well as Phylloplane fungi. In this method, the fungi grow on its own on the host, getting the moister produced from the wet condition prevailing inside the petriplates. All the plates were incubated at 25±3ºC temperature in the incubation chamber. Incubation time was maintained differently since, 7-8 days is meant for the fungal growth of fungi in agar plate method, but in moist chamber method, 1 to 3 weeks are required for the growth of fungi. Every day watch of the petriplates and check the growth of fungi was almost necessary in our present study after 3rd day of incubation.

Identification of fungi

After three days of incubation, the fungal colonies were counted for individual species and the total number was enumerated. Microscopic slides stained with lacto phenol cotton blue were prepared from each colony of the fungus and observed microscopically under the trinacular and digital photography microscope to identify up to species level. The colony which was not be identified directly from plates was sub cultured in SDA/PDA media again and identified later on. The laboratory experience and taxonomic literature were employed to identify the fungal CFUs up to species level [10,11,12,13]. The presence and absence based on the occurrence of individual fungus in the phylloplane and endophytic were determined and plotted in the form of tables and figures.

Results

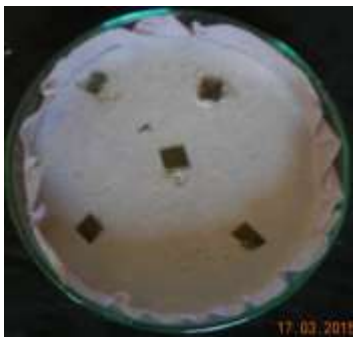
Growth of phylloplane and endophytic fungi in two types of technique plates are given in Fig 1. During the present study, altogether 16 fungal species under 10 genera were isolated and identified from the healthy leaf of the medicinal plant, *Solanum nigrum* by employing both agar plate and moist chamber methods. The plant was screened for the presence of phylloplane and endophytic fungi, of which 9 and 6 species of phylloplane and endophytic fungi were isolated and identified by agar plate method but by moist chamber method, 5 and 4 species of phylloplane and endophytic fungi were isolated and identified (Fig 2). Total number of endophytic and phylloplane fungi isolated from *Solanum nigrum* is given in Table 1. In agar plate method, *Alternaria* sp., *Cladosporium herbarum, Aspergillus awamori, A. flavipes, A. niger Monascus sp., Penicillium citrinum, P. digitatum, Torula herbarum* and *Trichoderma harzianum* were identified as phylloplane fungi and *Alternaria* sp., *Aspergillus niger*, *Aspergillus flavus*, Brown sterile mycelia, *Penicillium chrysogenum*, *P. digitatum, Torula herbarum, Trichoderma* sp. and White sterile mycelia were identified as endophytes. In moist chamber method *Aspergillus flavus*, *Curvularia lunata*, Brown sterile mycelia and *Trichoderma harzianum* were identified as phylloplane fungi, *Aspergillus flavus*, brown sterile mycelia and *Torula herbarum* were identified as endophytes. Out of all fungi recognized, *Torula herbarum* was found as the purest endophytes of the total number of isolated phylloplane and endophytic fungi recorded from the mature leaf of *Solanum nigrum* by two different methods are given in Table 1. Moist chamber method was found suitable to isolate the fungal species from the leaf samples of the medicinal plant. It was also observed that moist chamber was not expensive to prepare and to inoculate the materials like agar plate method. Moreover it was seen that the growth of endophytic and phylloplane fungi was very slow in the moist chamber than the agar plate method. All obligate parasitic or restricted fungi were found to grow in the moist chamber in better way than agar plates since they are likely to grow in their own host in the humidity condition than the agar plates where no humidity is prevailed.



Phylloplane fungi on agar plate

Endophytic fungi on agar plate

Phylloplane fungi on moist chamber plate



Endophytic fungi on moist chamber plate

Fig 1: Growth of phylloplane and endophytic fungi on agar and moist chamber plates.

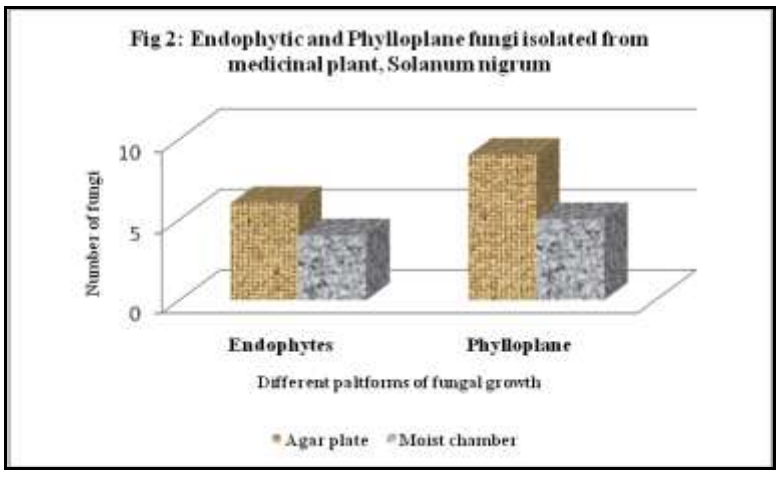


Table 1: Phylloplane and endophytic fungi isolated from *Solanum nigrum* by agar plate and Moist chamber plates.

Sl. No.	Name of the fungus	Agar plate method		Moist chamber method	
		Endophytes	Phylloplane	Endophytes	Phylloplane
1	*Alternaria* sp.	+	+	-	-
2	*Aspergillus awamori*	+	+	-	-
3	*Aspergillus flavipes*	-	+	-	-
4	*Aspergillus flavus*	-	-	+	+
5	*Aspergillus niger*	-	+	-	-
6	Brown sterile mycelia	-	-	+	+
7	*Cladosporium herbarum*	-	+	-	-
8	*Curvularia lunata*	-	-	-	+
9	*Curvularia* sp.	-	+	-	-
10	*Monascus* sp.	_	_	-	+
11	*Penicillium citrinum*	-	+	-	-
12	*Penicillium chrysogenum*	+	-	-	-
13	*Penicillium digitatum*	+	+	-	-
14	*Torula herbarum*	-	-	+	+
15	*Trichoderma harzianum*	+	+	+	+
16	White sterile mycelia	+	-	-	-

Discussion

In the present study the isolated fungi were belonged to the class Ascomycetes and Deuteromycetes. The colonization of the phylloplane and endophytic fungi has received considerable attention as they found to protect their host against pest, pathogen and even domestic herbivorous. These endophytes and phylloplane fungi may lead to production of special compound within the host plant. Fungi have been widely known as a source of bioactive compound; an excellent example for this is the anti cancer drug taxol, which was previously supposed to occur only in the plant tissues[14]. *Solanum nigrum* is a plant having a broad spectrum of medicinal properties. Every part of the plant is used in one or the other types of medicines. Isolation of only 16 taxa of phylloplane and endophytic fungi showed that the medicinal property of the plant has some role to play in the colonization of fungi. This low rate of colonization may be attributed to the secretion of the phyto-chemicals since they contain certain anti fungal and anti bacterial components. The data suggested that the smaller and the more scattered the plant fragment samples in order to reciprocate the number of fungal isolates. The higher the probability of approaching real diversity values of phylloplane and endophytic fungal communities. *Alternaria sp, Aspergillus awamori, Penicillium digitatum, Trichoderma harzianum,* isolated from *Solanum nigrum* is agreed with the previous workers who had also reported the same endophytic fungi in their study in agar plate[15]. Petrini[16] opined during 1986 that these common endophytes were being isolated frequently from leaves of medicinal plants. In moist chamber, *Aspergillus flavus*, Brown sterile mycelia, *Torula herbarum* and *Trichoderma harzianum* were isolated from the plant was quite unique in its finding.

Conclusion

During the present work, diversity of endophytic and phylloplane fungi was studied by agar plate and moist chamber techniques to isolate the endophytic and ectophytic fungi. During the course of work, a total of 16 fungi were isolated under 10 genera from both agar plate and moist chamber plates. Prevalence of the fungi isolated by moist chamber was found less than agar plates. In agar plate technique, *Penicillium digitatum, Aspergillus awamori, Alternaria* sp., recorded from the leaf samples but, in moist chamber, *Torula herbarum, Trichoderma harzianum* and *Aspergillus flavus* were recorded from the leaf samples. From the recorded fungi, *Torula herbarum* was found as the perfect endophytes among the total number of isolated phylloplane and endophytic fungi from the leaf samples of *Solanum nigrum* by two different methods. Moist chamber method was found to be easy to isolate endophytic and phylloplane fungi correctly in comparison to agar plate method, where the sporulation of fungi was very fast than the moist chamber.

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