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Comparative assessment of two methods for isolation of endophytic fungi from varied leaves of *Andrographis paniculata*

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Abstract: A comparative study pertaining to isolation and enumeration of endophytic fungi from young, mature, yellow, dry and infected leaf samples of a medicinal plant, *Andrographis paniculata* was carried out in the Microbiology Laboratory, Department of Botany, K. M. Centre for P. G. Studies (Autonomous), Pondicherry, India. Agar plate and moist chamber methods were used to isolate the endophytic fungi. In *Andrographis paniculata*, altogether 23 fungal species were isolated under 17 genera from both Agar plate and moist chamber methods. Of which 15 species of 14 genera were recorded from Agar plate and 13 species of 12 genera were recorded from moist chamber. It was found that the *Botrytis cinerea* was recorded in all the leaf samples started from young to infected (except yellow in Agar) of the medicinal plant. In Agar plate, *Botrytis cinerea*, *Wallemia sebi*, White sterile mycelia were recorded maximum in all the leaf samples. In Moist chamber, *Aspergillus niger*, *Botrytis cinerea* were recorded in all the leaf samples starting from young to infected. Most of the fungal species were found in all the leaf samples viz., *Alternaria alternata*, *Aspergillus niger*, *Wallemia sebi*, White sterile mycelia, *Botrytis cinerea*, *Cercospora heteromella*, *Curvularia lunata* and *Trichoderma* sp. The fungi like *Aspergillus niger, Cercospora heteromella, Curvularia lunata* were predominant in the Moist chamber method. Agar plate method was found suitable to isolate and record the endophytic fungi correctly in comparison to moist chamber method. The similarity coefficient value was 50% in between the moist chamber and agar plate methods based on their fungal diversity and common number species.
Keywords: Endophytic fungi, *Andrographis paniculata*, Moist chamber method.

Introduction

Endophytic microbes, particularly fungi that live inside plant tissues without making any symptoms of disease to the host[1]. Clay[2] described that natural ecosystems are infested by fungi without any external manifestation of disease other than endophytic fungi. The term 'endophyte' has been controversial since its appearance[3] and there are various reports that endophytes can become parasites under certain conditions[4]. The colonization of plant tissues by endophytic fungi occurs in a manner similar to those of plant pathogens and mycorrhizae colonization comprises a sequence of steps involving host recognition by the fungus, spore germination, penetration of the epidermis and tissue colonization[5]. They are a large number of unexplored plant communities whose endophytic fungal biodiversity was not known, especially in the tropics. In recent days many antimicrobial compounds are being produced by endophytic fungi in culture and they are proven as effective against plant and human pathogenic microorganisms. Number of works pertaining to endophytic fungi of different medicinal plants in and around India was carried out by various workers, but there is no work in the same field in Puducherry State. It is necessary to find out the patterns of distribution of endophytic fungi from different medicinal plants as well as the their succession adhered to the leaves based on the ageing of the plant

and to recognise the fungi related to the metabolites produced from these medicinal plants. It is an attempt to isolate endophytic fungi that are specific to *Andrographis paniculata.* During the present study, I have isolated and enumerated the endophytic fungi by employing two techniques from the leaves of *Andrographis paniculata* collected from Tagore Arts College and K M Centre for PG Studies (Autonomous) campus, Puducherry, India.

Materials and Methods

Andrographis paniculata, Family: Acanthaceae

Andrographis paniculata is an erect annual herb extremely bitter in taste through out the plant body. The plant is famous in north-eastern India as *Maha-tita*, literally as king of bitters. As an Ayurveda herb it is known as *Kalmegh* or *Kalamegha*, meaning; dark cloud. It is also known as *Bhui-neem*, meaning; neem of the ground. Since the plant though being a small annual herb has a similar strong bitter taste as that of the large Neem tree (*Azadirachta indica*). In Malaysia, it is known as *Hempedu Bumi*, which literally means 'bile of earth.' As it is one of the bitterest plants hence are used in traditional medicine. The genus *Andrographis* consists of 28 species of small annual shrubs essentially distributed in tropical Asia. Only a few species are medicinal, of which *A. paniculata* is the most popular.

Medicinal uses

Since the ancient times, *A. paniculata* is used in traditional Siddha and Ayurvedic systems of medicine as well as in tribal medicine in India and some other countries for multiple clinical applications. The therapeutic value of Kalamegha is due to its mechanism of action which is perhaps by enzyme induction. The plant extracts exhibits anti-typhoid and antifungal activities. Kalmegh is also reported to possess antihepatotoxic, antibiotic, antimalarial, antihepatitic, antithrombogenic, antinflammatory anti-snake venom and antipyretic properties, besides its general use as an immunostimulant agent. The results suggest that andrographolide is an interesting pharmacophore with anticancer and immunomodulatory activities and hence has the potential for being developed as a cancer therapeutic agent.

Collection of plant samples

Leaf varieties based on age viz., young, mature, yellow, infected and dry of the medicinal plant, *Andrographis paniculata* were carefully chosen, collected from Tagore Arts College and K M Centre for PG Studies (Autonomous) campus, Pondicherry and brought to the Microbiology Laboratory, Department of Botany in aseptic condition and kept in the refrigerator at 4-8^{o}C up to the completion of the experiment.

Isolation of endophytic fungi

The leaf samples were rinsed gently in running tap water to remove dusts and debris. The leaves were cut into segments (0.5 – 1cm). The samples were immersed in 70% ethanol for 5 seconds, followed by 4% sodium hypochlorite for 90 seconds and then rinsed in sterile distilled water for 10 seconds/ three times in a way. The excess moisture was blotted in a sterile filter paper. The surface sterilized segments were placed in Petridishes containing PDA medium as well as in moist chamber plates. The Petridishes were sealed using parafilm and incubated at 25 ± 3^{o}C at 12-h light/dark cycle. After incubation of three day, the Petridishes were monitored every day to check the growth of endophytic fungal colonies from the segments and were identified separately based on the availability of Laboratory manuals and references[6,7,8]. The sterile endophytes i.e., the non-sporulating sterile forms that could not be assigned to any taxonomic group were given separate numbers and maintained in pure cultures. They were distinguished from each other by their cultural characteristics such as colony morphology, growth rates, hyphal mat characteristics and pigmentation of the fungal colony and medium. All the endophytic isolates were documented and maintained in PDA slants. Tables and figures were made based on the presence and absence of endophytic fungi on leaf samples. Growth of endophytic fungi on agar plate and moist chamber are given in Plate I.

Agar Plate **Moist chamber**

Plate I: Growth of endophytic fungi on agar plate and moist chamber

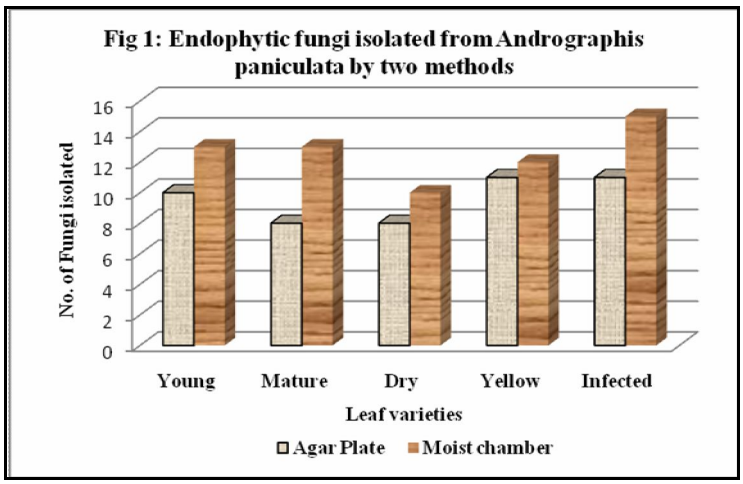
Results and Discussion

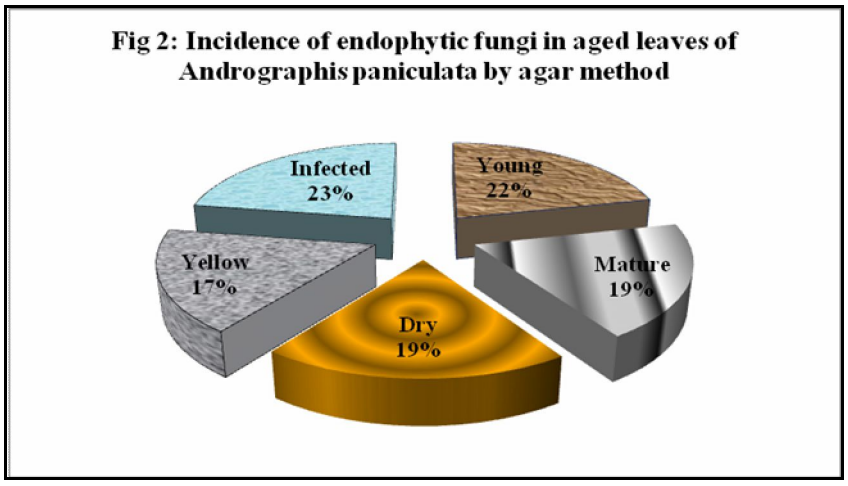
During the study period, altogether 23 fungal species were isolated under 17 genera from both Agar plate and Moist chamber methods from the varietal leaves of *Andrographis paniculata*. Of which 15 species of 14 genera were recorded from Agar plate and 13 species of 12 genera were recorded from Moist chamber. Endophytic fungi isolated from *Andrographis paniculata* in Agar plate and Moist chamber methods was given in Table 1. It was found that the *Botrytis cinerea* was recorded in all the leaf samples started from young to infected (except yellow in Agar) of the medicinal plant *Andrographis paniculata*. In agar plate (Table 1) *Botrytis cinerea*, *Wallemia sebi*, White sterile mycelia were recorded maximum number in all the leaf samples. In Moist chamber *Aspergillus niger*, *Botrytis cinerea* were recorded in all the leaf samples starting from young to infected. Most of the fungal species were found in all the leaf samples viz., *Alternaria alternata*, *Aspergillus niger*, *Wallemia sebi*, White sterile mycelia, *Botrytis cinerea*, *Cercospora heteromella*, *Curvularaia lunata* and *Trichoderma* sp. The fungi like *Aspergillus niger, Cercospora heteromella, Curvularia lunata* were predominant in the Moist chamber method. Total number of endophytic fungi isolated from different leaf samples of *Andrographis paniculata* by agar plate and Moist chamber method is given in Fig.1 which showed that young and infected leaves of the plant harboured maximum number of endophytic fungi followed by mature and yellow leaf. Dry leaf contains only less number of fungi. In the agar plate, only young leaf contained more number of endophytic fungi followed by infected, yellow, mature and dry respectively. Like moist chamber method mature leaf showed maximum number of fungi followed by infected and young. Percentage occurrence of endophytic fungi recorded in different leaves of the medicinal plant, *A. paniculata* is given in Fig 2.

Table 1: Occurrence of endophytic fungi isolated from *Andrographis paniculate* by agar plate and moist chamber method.

Sl. No.	Endophytic fungi	Leaf samples									
		Young		Mature		Dry		Yellow		Infected	
		Ag	Mc	Ag	Mc	Ag	Mc	Ag	Mc	Ag	Mc
1	Alternaria alternata	+	+	+	+	+	+	-	+	-	+
2	A. niger	-	+	-	+	-	+	+	+	+	+
3	Aspergillus sp.	-	-	-	-	-	-	-	+	-	+
4	Botrytis cinerea	+	+	+	+	+	+	-	+	+	+
5	Cercospora heteromella	+	+	-	+	-	+	+	+	+	+
6	Cladosporium herbarum	+	-	-	+	+	-	+	+	+	+
7	Colletotrichum sp.	-	+	+	+	-	-	+	-	+	+
8	Curvularia lunata	-	+	-	+	-	+	-	+	+	+
9	C. geniculata	+	-	+	-	-	-	+	-	-	-
10	Drechslera sp.	-	+	-	-	-	+	-	-	-	+
11	Gliocladium sp.	+	-	-	-	+	-	+	-	+	+
12	Green sterile mycelia	-	-	+	-	+	-	+	-	-	-
13	Penicillium citrinum										
14	Humicola sp.	-	+	+	+	+	+	-	-	-	+
15	Penicillium citrinum	-	+	+	-	-	-	-	-	-	+
16	Pink sterile mycelia	+	+	-	+	-	-	-	+	+	-
17	P. italicum	-	-	-	+	-	+	-	+	-	-
18	Saccharomyces sp.	+	+	-	+	-	+	+	+	-	+
19	Trichoderma sp.	-	+	-	+	-	+	-	+	-	+
20	Ulocladium sp.	+	-	-	-	-	-	-	-	+	-
21	Volutella buxi	+	-	+	-	-	-	+	-	-	-
22	Wallemia sebi	-	-	+	-	+	-	+	-	+	-
23	White sterile mycelia	-	+	-	+	+	-	+	+	+	+

Ag: Agar plate; Ms: Moist chamber





Occurrence of maximum number of endophytic fungi in young and infected leaves comparison to mature and yellow leaves may be due to the availability of more nutrients in the concerned leaves of the host plant. Agar plate method was suitable to isolate and record more numbers may be due to its host specific nature and suitable composition of media for growth of fungi. It was easy to identify fungal isolates in moist chamber since they were likely to grow in their own host in the moist chamber's humidity condition. White sterile mycelia and *Curvularia* were predominant in the agar plate method, but in moist chamber technique, *Colletotrichum* sp., *Curvularia*, *Penicillium citrinum* and white sterile mycelia were predominant. *Volutella* sp. and *Wallemia* sp. were isolated specifically by agar plate method and aspergilli, *Drechslera* sp. and *Trichoderma* sp. were restricted to moist chamber may be due to media suitability of these fungi to grow within the plant. Different fungi emerged from the leaf segments indicating that segments may be occupied by more than one fungus. In our study, particularly to various leaf samples from the medicinal plant, *Andrographis paniculata* of Pondicherry U.T. region was screened for diversity and composition of endophytic fungal communities is equivalent to the previous works made by others [9,10]. This data suggested that the smaller and the more scattered the plant fragments sampled, the higher the probability of approaching real diversity of endophytic fungal communities. *Alternaria, Aspergillus, Cladosporium* isolated from *Andrographis paniculata* was agreed with the previous workers who had also reported the same endophytic fungi in their study[1,9]. Petrini and Carroll[10] reported that *Alternaria* spp, *Cladosporium* spp were not host specific fungi, but they used to be recorded from most of the plants. Certain endophytic fungi may be highly host specific while others are generally distributed[11]. Petrini and Carroll[10] contended that fungal endophytes exhibit some degrees of host specificity at least for families of host plant and that this specificity determines endophytic distribution more than the geographic location of the host plant. The incidence of the endophytic fungi is influenced by the age of leaf tissues and their colonization frequency and species richness increase with the age of the plant[1,10], which was proved in our study since the endophytic flora generally increased according to the aging of the leaves. Endophytes were now considered as an outstanding source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments[2,12].

Conclusion

Enumeration of endophytic fungi isolated from different leaf samples of medicinal plant, *Andrographis paniculata* by two methods showed that the infected and young leaves of the plants harbored maximum number of endophytic fungi followed by yellow and mature. Agar plate method was found suitable for isolation and enumeration of endophytic fungi in comparison to moist chamber, but moist chamber was easy to assess and to identify fungi more accurately than the other one.

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