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Endophytic fungal enumeration from various leaf samples of a medicinal plant: Ziziphus mauritiana

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Abstract: Isolation and enumeration of endophytic fungi from different leaf samples viz., young, mature, dry, yellow and infected of *Ziziphus mauritiana* was carried out in the Microbiology laboratory, K. M. Centre for P. G. Studies (Autonomous), Pondicherry during 2011. Agar plate and moist chamber techniques were used to isolate the endophytic fungi. During the study period, a total of 16 fungi were isolated under 10 genera from both agar plate and moist chamber method of which, 13 species of 10 genera were from Agar plate and 13 species of 9 genera were from moist chamber. Incidence of endophytic fungi isolated from *Ziziphus mauritiana* in agar plate was less than moist chamber. It was found that, white sterile mycelia was recorded in all the leaf samples starting from young to infected of the medicinal plant *Ziziphus mauritiana.* In agar plate technique, Aspergilli*, Cladosporium cladosporioides, Penicillium citrinum,* white sterile mycelia, *Curvularia lunata*, gray sterile mycelia were recorded from all the leaf samples. In moist chamber, *Fusarium oxysporum, Gliocladium* sp*., Humicola* sp. and white sterile mycelia were recorded in all the leaf samples. It showed that infected and yellow leaves of the plant harboured maximum number of endophytic fungi followed by mature and young leaves. Moist chamber method was suitable to isolate and record the endophytic fungi correctly in comparison to agar plate method.

Keywords: Medicinal plant, Endophytic fungi, *Ziziphus mauritiana*, Moist chamber method.

Introduction

Fungi are morphologically diverse in nature, they occur in wide spectacular array of shapes, sizes and colors over numerous substrates. Fungi who live endophytically inside the tissues of living plants are under-explored group of microbes with regards to their involvement in the production of noble metabolites. During early nineties, Dreyfuss and Chapela[1] estimated that there may be at least one million species of endophytic fungi alone available in the world wide. During the recent days they have received considerable attention. They were recognized to protect their host against insect pests, pathogens and even domestic herbivores[2,3]. Medicinal plants are reported to harbor endophytes[4], which in turn provide protection to their host from infectious agents and also provide adaptability to survive in adverse environmental conditions. To date, only a few plants have been extensively investigated for their endophytic biodiversity. A number of works pertain to endophytic fungi of different medicinal plants in and around of India were carried out by various workers, but there is no work in the same field in Puducherry State. It was necessary to establish the patterns of distribution of endophytic fungi from different leaf samples of *Ziziphus mauritiana* as well as the succession of endophytic fungi adhered to the leaves based on the ageing of the plant as well as to recognise the fungi related to the metabolites produced from this medicinal plant. The ultimate interest was to isolate widespread fungi that may be specific *Ziziphus mauritiana.* Based on the discussion above, the present work is an attempt to screen out and identify the endophytic fungi by employing two techniques from one medicinal plant, *Ziziphus mauritiana* collected from TAC and KMCPGS campus, Puducherry, India.

Materials and Methods

Zyziphus marizuana: a medicinal plant

Ziziphus mauritiana, known as Ber, Chinee Apple, Jujube, Indian plum and Masau is a tropical fruit tree species belongs to family Rhamnaceae. *Z. mauritiana* is a spiny, evergreen shrub or small tree up to 15 m high, with trunk 40 cm or more in diameter; spreading crown; stipular spines and many drooping branches. The fruit is of variable shape and size. It can be oval, obovate, oblong or round, and that can be 1-2.5 in (2.5-6.25 cm) long, depending on the variety. The flesh is white and crisp. When slightly under ripe, this fruit is a bit juicy and has a pleasant aroma. The fruit's skin is smooth, glossy, thin but tight. The species is believed to have originated in Indo-Malaysian region of South-East Asia. It is now widely naturalized throughout the Old World tropics from Southern Africa through the Middle East to the Indian Subcontinent and China, Indo-Malaya, and into Australasia and the Pacific Islands. The fruit is eaten raw, pickled or used in beverages. It is quite nutritious and rich in vitamin C. It is second only to guava and much higher than citrus or apples. In India, the ripe fruits are mostly consumed raw, but are sometimes stewed. Slightly under ripen fruits are candied by a process of pricking, immersing in a salt solution. Ripe fruits are preserved by sun-drying and a powder is prepared for out-of-season purposes. It contains 20 to 30% sugar, up to 2.5% protein and 12.8% carbohydrates. Fruits are also eaten in other forms, such as dried, candied, pickled, as juice or as ber butter. The fruits are applied on cuts and ulcers; are employed in pulmonary ailments and fevers; and, mixed with salt and chili peppers, are given in indigestion and biliousness. The dried ripe fruit is a mild laxative. The seeds are sedative and are taken, sometimes with buttermilk, to halt nausea, vomiting, and abdominal pains in pregnancy. They check diarrhea, and are poulticed on wounds. Mixed with oil, they are rubbed on rheumatic areas. The leaves are applied as poultices and are helpful in liver troubles, asthma and fever and, together with catechu, are administered when an astringent is needed, as on wounds. The bitter, astringent bark decoction is taken to halt diarrhea and dysentery and relieve gingivitis. The bark paste is applied on sores. The root is purgative. A root decoction is given as a febrifuge, taenicide and emmenagogue, and the powdered root is dusted on wounds. Juice of the root bark is said to alleviate gout and rheumatism. Strong doses of the bark or root may be toxic. An infusion of the flowers serves as an eye lotion.

Collection of plant samples

The leaf samples viz., young, mature, infected, yellow and dry of the medicinal plant, *Ziziphus mauritiana* were carefully chosen, collected from the KMCPGS and TAC 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 (Plate I).

Young	Mature	Yellow	Dry	Infected

Plate I: Different leaf samples of Ziziphus mauritiana collected for isolation of endophytic fungi.

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 surface sterilized by modified method [5]. 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]. 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.

Results

Altogether, 16 species were isolated under 10 genera from both Agar and Moist method from *Ziziphus mauritiana.* Of that, 13 species of 10 genera from Agar plate and 13 species of 9 genera from Moist chamber method. Incidence of endophytic fungi isolated from *Ziziphus mauritiana* in Agar Plate and Moist chamber is given in Table 1. It was found that *Aspergillus flavipes* was recorded almost all leaf samples of Agar plate method. *Curvularia geniculata*, *Aspergillus fumigatus* were very less in the entire sampled leaves. In Moist chamber *Cladosporium cladosporioides* was more prominent followed by *Fusarium oxysporum* and *Saccharomyces* sp. *Aspergillus niger* was isolated in very less number. Total number of endophytic fungi isolated from different leaf samples of *Ziziphus mauritiana* by two methods is given in Fig 1. It revealed that infected and dry leaves showed the maximum number of endophytic fungi followed by young, mature and yellow. In Moist chamber young leaves showed the maximum number followed by mature and yellow leaves.

Table 1: Occurrence of endophytic fungi isolated from *Zizyphus mauritiana* 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	Aspergillus flavus	-	-	+	-	-	-	+	-	+	-
2	A. flavipes	+	+	+	+	-	+	+	+	+	-
3	A. fumigatus	-	+	-	+	-	-	-	-	-	+
4	A. niger	+	+	+	+	+	-	-	-	-	-
5	Cladosporium cladosporioidies	-	+	-	+	+	+	+	+	+	+
6	Curvularia lunata	-	+	-	+	+	-	-	+	+	+
7	C. geniculata	-	-	-	-	-	-	-	-	+	-
8	Fusarium oxysporum	-	+	-	+	-	+	-	+	+	+
9	Grey sterile mycelia	-	+	+	-	+	+	-	-	+	+
10	Gliocladium sp.	-	+	+	+	+	-	-	+	-	-
11	Humicola sp.	-	+	+	+	+	+	-	-	-	+
12	Penicillium citrinum	+	-	-	-	+	+	-	+	+	+
13	P. fellutanum	+		-		-		+		+	
14	P. italicum	-	-	-	+	-	+	-	+	-	-
15	Saccharomyces sp.	+	+	-	+	-	+	+	+	-	+
16	White sterile mycelia	-	+	-	+	+	-	+	+	+	+

Ag: Agar plate; Ms: Moist chamber

Total number of endophytic fungi isolated from different leaf samples of *Ziziphus mauritiana* by two methods is given in Fig 1. It showed that infected and yellow leaves of the plant harbored maximum number of endophytic fungi followed by mature and young leaves. Moist chamber method was suitable to isolate and record the endophytic fungi correctly in comparison to agar plate method. 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.

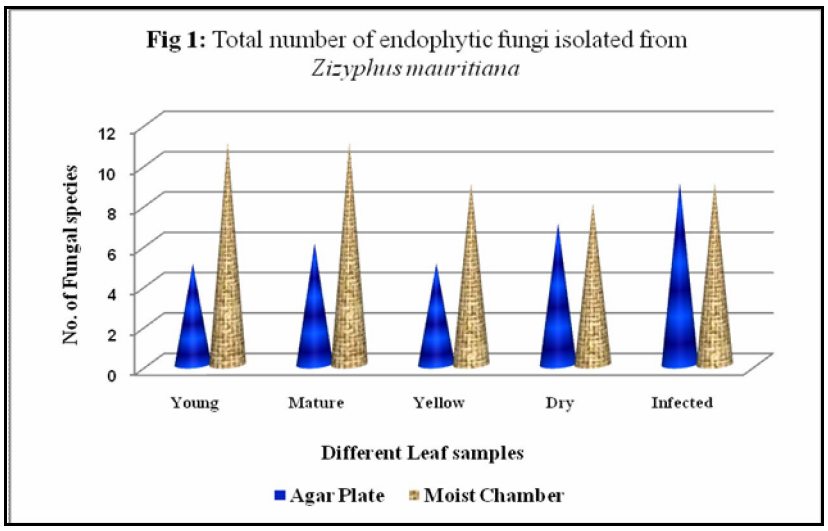

Fig 1: Total number of endophytic fungi isolated from *Zizyphus mauritiana*

Discussion

In the present study, Different fungi emerged from the leaf segments indicating that segments may be occupied by more than one fungus. Fungi that emerge late were found usually slow growing, were discarded prior to their detection. In this study, in particular to the various leaf samples from the medicinal plant, *Ziziphus mauritiana* of Pondicherry region was screened for diversity and composition of endophytic fungal communities is equivalent to the previous work made by Santhosh and his corkers in the coastal region of Arabian sea[8]. The data suggested that the smaller and the more scattered the plant fragments sampled, the higher the probability of approaching real diversity values of endophytic fungal communities. *Alternaria alternata, Aspergillus, Cladosporium* spp isolated from *Ziziphus mauritiana* was agreed with the previous workers who had also reported the same endophytic fungi in their study[8]. These common endophytes were isolated frequently from the leaves of medicinal plant. Petrini and Carroll[9] reported that *Alternaria* spp, *Cladosporium* spp were not host specific fungi, but they used to be recorded from tissues of different host plants. Certain endophytic fungi may be highly host specific while others are generally distributed[10]. Petrini and Carroll [9] 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 occurrence of the endophytes is influenced by the age of leaf tissues[9]. Generally their colonization frequency and species richness increase with the age of the plant tissue[10], which was proved in our study since the endophytic flora generally increased according to the aging of the leaves. Studies on endophytic microbes over the last two decade indicate that they occupy a unique ecological niche and are thought to influence plant ecology and its distribution[11]. 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[12]. In the present study, Moist chamber method was found suitable to isolate systemic endophytic fungi correctly from the palnt comparison to agar plate method.

Conclusion

Endophytic fungi isolated from different leaf samples of medicinal plant, *Ziziphus mauritiana* plant by two methods showed that the infected and dry leaves of the plants harbored maximum number of endophytic fungi followed by yellow, mature and young. Moist chamber method was found suitable for isolation and enumeration of endophytic fungi correctly in comparison to agar plates. The similarity coefficient value was found maximum in moist chamber method than the agar plate method.

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