ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 12, pp. 14580-14583, December, 2016

International Journal of Recent Scientific Research

Research Article

ISOLATION, IDENTIFICATION AND FREQUENCY STUDIES OF FOLIAR ENDOPHYTIC FUNGI FROM DENDROBIUM SP. AND ONCIDIUM SP

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ARTICLE INFO

Article History:

Received 15th September, 2016
Received in revised form 25th October, 2016
Accepted 28th November, 2016
Published online 28th December, 2016

Key Words:

Orchids, *Dendrobium*, *Oncidium*, Endophytes, Colonization frequency.

ABSTRACT

In the present investigation, a total of 47 isolates belonging to 8 genera of foliar endophytic fungi were isolated from two orchids- *Dendrobium*sp. and *Oncidium* sp. They were identified to 8 genera which belonged to the class Ascomycetes (5 genera), Basidiomycetes (1 genera) and 2 sterile mycelium. *Dendrobium* sp. and *Oncidium* sp. showed the presence of 4 and 5 genera of endophytic fungal genera respectively. Maximum colonization frequency and relative abundance was shown by *Curvularia* sp. and *Cercospora* sp. in *Dendrobium* sp. and *Oncidium* sp. respectively. Maximum species diversity was shown by *Oncidium* sp. than *Dendrobium* sp. Similarity index of both orchids were also calculated.

INTRODUCTION

Orchids are monocotyledonous plants and taxonomically belong to Order Orchidales and Family Orchidaceae. There are 15000 to 25000 of orchid species distributed all around the world, mostly can found in tropical environment. They all can divide in 2 classifications as epiphytic orchids and terrestrial orchids. The special hereditary characteristic of orchid species is having many tiny seed in the pod and no endosperm in embryo. So to be geminated, the orchids have to depend on some fungi species to be the energy and food sources such as many nutrients that are important for growth (Thamasiri K., 2005).

Plants are usually associated with diverse microorganisms. Endophyte microorganisms are those that colonize the healthy plant internal tissue (Stone J.K. *et al*., 2004). The meaning of term "endophyte" is as broad as its literal definition and spectrum of potential hosts and inhabitants. The word 'Endophytes' are used for both bacteria and fungi (Schulz, B. *et al*., 2006). Modern usage of the term endophytic fungi in mycology refers to those fungi which live within leaves stems roots and other part of apparently healthy host plants and can not be seen in visually signs of infection (Stone J.K. *et al*., 2004). Dreyfuss and Chapela (1994) estimated that there may be at least one million species of endophytic fungi alone. It means that almost all plant species are usually infected with endophytic fungi.

Orchids with horticultural, ornamental, medical and commercial importance have been studied for the presence of endophytes. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites such as mycotoxins, enzymes and antibiotic (Zhang *et al*., 2006). Those fungi can be found in the root, stem, leaf, flower, fruit and seed, without any disease or impairness showed by the host.

The objective of this study is to isolate, identify and study the frequency of foliar endophytic fungi of 2 orchids- *Dendrobium* sp. and *Oncidium* sp. Since endophytes are commonly defined as 'all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host' (Petrini, 1991) only healthy organs were used in these studies.

MATERIALS AND METHODS

Collection of Plants materials

The study of endophytic fungi starts with a collection of orchid samples, followed by isolation in the laboratory. Five healthy and mature plants of *Dendrobium* sp. and *Oncidium* sp. each were chosen for sampling from Orchidarium of Assumption College, Changanacherry during the period of October – November 2016. Two mature asymptomatic leaves from each plant was collected and bought to the laboratory in sterilized bags and processed within few hours after sampling.

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Isolation of endophytic fungi

The leaves were gently rinsed in freshwater to remove extraneous matter. The surface sterilization was done by procedure used by Blumenstein (2010) with some modifications. Symptomless leaves of each plant were rinsed in a sequence distilled water for 1 min, 75% ethanol for 30s, 0.1% $HgCl_2$ for 1 min, and 75% ethanol for 30s and then rinsed in sterile distilled water three times. Leaf imprinting was done to test the effectiveness of the protocol (Schulz *et al.*, 1993). Leaves were then cut into pieces without any midrib under aseptic conditions. Two pieces each from one leaf were taken and thus a total of 20 pieces of leaves from each plant were inoculated in Potato dextrose agar medium supplemented with antibiotic ambistrynand incubated at 28ºC for 7 days and periodically checked for purity. The fungal strains in the pure culture were preserved on PDA slant at 4ºC with proper labelling. The isolates were subcultured on potato dextrose agar for morphological identification.

Identification of fungal endophytes

For characterization of the morphology of fungal isolates, slides were prepared from cultures and stained with cotton blue reagent in lacto phenol and examined with a compoundmicroscope. Identification was based on morphological characteristics such as growth pattern, colony and hyphae, colour of colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and characteristics of the spore (H. L. Barnett and B. B. Hunter, 1956).

Data analysis

Colonization frequency

Colonization frequency (%) of each fungus on orchid segments were estimated:

Colonization frequency (%) of an endophyte species was equal to the number of segments colonized by a single endophyte divided by the total number of segments observed x100

Relative abundance

Relative abundance (%) of each endophytic fungal species was calculated as number of isolates of each fungal species divided by total number of endophytic fungal isolates x 100 (Kharwar *et al*., 2008).

Species diversity

The diversity of endophytic fungi in these 2 orchids were assessed on the basis of Brillouin diversity index (Stiling, 2012).

$$HB = \frac{\ln N! - \Sigma \ln n!}{N}$$

Where,
N = Total no. of individuals of all species in the community
n = No. of individuals in each species

Similarity index

To evaluate the degree of community similarity of endophyticspecies between two plants, Sorenson's coefficient (*CS*) was employed and calculated according to the following formula:

$CS = 2j / (a+b)$

where*j* is the number of endophytic species coexisted in both plants, *a* is the total number of endophytic species in one plant, and *b* is the total number of endophyticspecies in the other plant.

RESULTS AND DISCUSSION

In the present investigation, foliar endophytic fungi were isolated and identified from 2 different orchids – *Dendrobium* sp. and *Oncidium* sp. Epiphytic microorganisms were removed via surfacesterilization prior to isolation. The degree of surface sterilization greatly affects the fungal endophytes recovered. However, in most orchid endophyte studies to date, leaf imprinting was not carried out. The work of Sawmya *et al* (2013) was the only orchid endophyte study that tested the effectiveness of their surfacesterilization protocol. No microorganisms grew on media after imprinting the surface-sterilized tissues on agar, which indicated that their surface sterilization protocol was successful.

Table 1 List of foliar endophytic fungal genera isolatedfrom *Dendrobium* sp. and *Oncidium* sp.

Ascomycetes	Basidiomycetes	
1. *Aspergillus* sp. 2. *Cercospora*sp. 3. *Curvularia*sp. 4. *Cylindrocarpon*sp. 5. *Fusarium* sp.	1. *Rhizoctonia* sp.	*Mycelia sterilia* 1 *Mycelia sterilia* 2

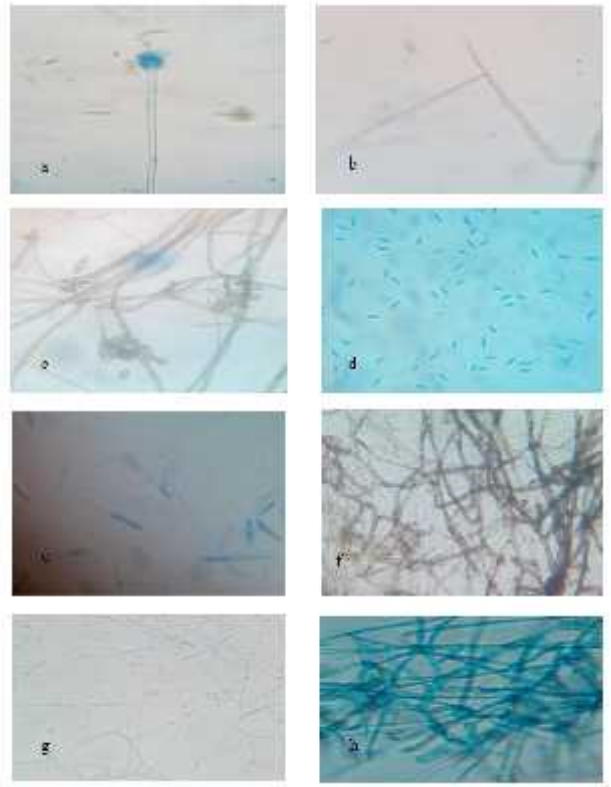


Figure 1 a. *Aspergillus* sp. b. *Cercospora* sp. c. *Curvularia* sp. d. *Cylindrocarpon* e. *Fusarium* f. *Rhizoctonia* g. *Mycelia sterilia* 1 h. *Mycelia sterilia* 2

A total of 47 isolates of foliar endophytes were obtained from the two orchids. They were identified to 8 genera which belonged to the class Ascomycetes (5 genera), Basidiomycetes (1 genera) and 2 sterile mycelium (Table 1) (Fig 1).Worldwide,

the fungi involved with orchids are almost all members of the phylum Basidiomycota group, however many do not produce sexual spores, and are consequently assigned to the form genus *Rhizoctonia* (Rasmussen., 2002). Although results may vary with the species of orchid examined, it appears that under natural conditions a particular orchid species may associate with only a few or a single fungal species.

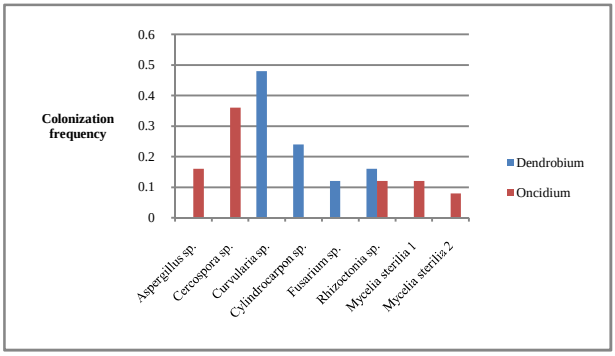
Dendrobium sp. and *Oncidium* sp. showed the presence of 4 and 5 genera of endophytic fungal genera respectively (Table 2).

Table 2 Fungal assemblage of endophytic fungi of 2 orchids (*Dendrobium* sp. and *Oncidium* sp.)

Orchid	Assemblage				
	Sa	Sco	Fi	Fts	Total genera
Dendrobium sp.	20	18	25	1.3	4
Oncidium sp.	20	19	22	1.1	5

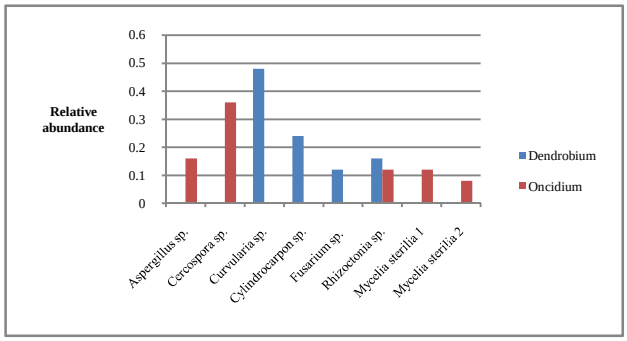
Sa: No. of segments assessed; Sco: No. of segments colonized; Fi: Total fungal isolations; Fts: No. of fungal taxa per segment.

Colonization frequency of the each fungal genera isolated from the two orchids were calculated (Graph 1). *Curvularia*sp. and *Cercospora* sp. showed maximum colonization frequency in *Dendrobium* sp. and *Oncidium* sp. respectively. *Rhizoctonia* sp. was common to both orchids. Okane *et al*. (1998) reported that the composition and frequency of a colonization related with the place and the host condition. Araujo *et al*. (2002) recorded that community of endophytic fungi depends on the interaction of microbial endophytic or any other pathogen. The existence of endophytic fungi is influenced by the variation of the season the environmental factors and the type of its host tissue (Rodrigues., 1994).



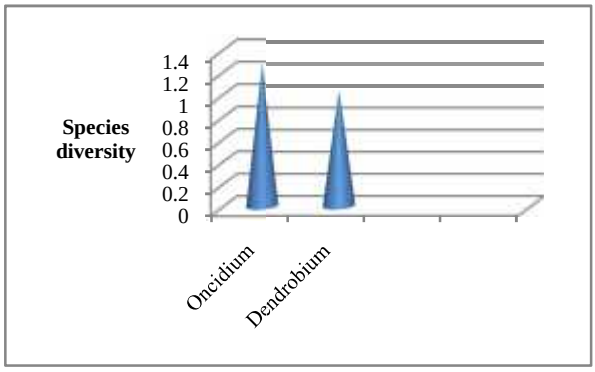
Graph 1 Colonization frequency of fungal genera isolated

Relative abundance of each endophytic fungal genera was calculated (Graph 2).*Curvularia* sp. and *Cercospora* sp. showed maximum relative abundance in *Dendrobium* sp. and *Oncidium* sp. respectively.



Graph 2 Relative abundance of fungal genera isolated

Species diversity of endophytic fungal genera isolated were calculated. Maximum species diversity was shown by *Oncidium* sp. (1.28) followed by *Dendrobium* sp. (1.05) (Graph 3).The diversity of orchid non-mycorrhizal endophytic fungi probably also depends on the localities from where the orchids were collected.



Similarity index of the endophytic fungal community of the two orchids was calculated and showed a similarity of 0.222 (Table 3). Sudheep and Sridhar (2012) reported that relatively similar endophytic fungal assemblages were isolated from distantly related orchids *Vandatestace* and *Bulbophyllumneilgherrense* sampled in the same habitat, i.e. the Kaiga forest of the Western Ghats, India. Shefferson *et al.* (2008) reported that there was no overlap in taxa of non-mycorrhizal endophytic fungi isolated from individuals of *Epipactisatrorubens* sampled respectively, at a meadow in a coastal farm and at Ash Hill.

Table 3 Similarity index of the endophytic fungal community of two orchids

	***Dendrobium* sp.**	***Oncidium* sp.**
Dendrobium sp.	1	0.222
Oncidium sp.		1

Endophytes inhabiting leaves (short-lived, photosynthetically versatile, and subject to damage by herbivores) are under high selective pressure, compared to those fungi associated with persistent tissues (bark, xylem or other woody parts) (Arnold., 2007a). Roles of non-mycorrhizal endophytic fungi in orchids need further investigation. In addition to investigation of roots, stems, and leaves, future studies on orchid endophytes need to evaluate seeds and inflorescences, which might be helpful to understand the role of endophytic fungi in orchid biology, cultivation, conservation, and natural product recovery (Sudheep and Sridhar., 2012).

Acknowledgement

We express our sincere thanks to Dr.Neeta. N.Nair, Associate Professor, Department of Botany, Mar Thoma College, Thiruvalla for helping me in the process of identification of the isolated fungal genera.

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How to cite this article:

Hina Mohamed and Marykutty Joseph.2016, Isolation, Identification and Frequency Studies of foliar endophytic Fungi from Dendrobium sp. And Onc idium SP. *Int J Recent Sci Res.* 7(12), pp. 14580-14583.