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Endophytic fungi of orchids of Arunachal Pradesh, North Eastern India

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Abstract

The leaf and root tissues of eleven orchid species occurring in Arunachal Pradesh, north eastern state of India were screened for their endophyte assemblages. The diversity of endophytes was higher in the leaves when compared to that in the roots. The endophytes exhibited tissue specificity and there was little overlap between the leaf and root assemblages. Species of *Xylaria* were ubiquitous; they were isolated from both roots and leaves and were dominant in the roots of eight of the eleven orchids. It is suggested that traits such ability to survive as saprotroph in plant litter, production of plant cell wall degrading enzymes for litter substrate utilization and elaboration of antimicrobial compounds for effective competition result in the niche expansion of Xylariaceous fungi to occupy both root and leaf tissues as endophytes.

Key words – leaf endophyte – root endophyte – *Xylaria*

Introduction

Endophytes are asymptomatic fungi residing in aerial and underground tissues of plants. Endophytes are the subject of bioprospecting exercise owing to their ability to produce novel bioactive compounds (Suryanarayanan et al. 2009) and industrial enzymes (Suryanarayanan et al. 2012, Thirunavukkarasu et al. 2015), as well as their capacity to reduce abiotic (Redman et al. 2002) and biotic stress in plants harbouring them (Estrada et al. 2015). Orchidaceae, with around 900 genera and more than 27,000 species, is one the most specious families of angiosperm. According to the recent review of Ma et al. (2015), only about 200 genera of orchids have been investigated for their non-mycorrhizal endophytic fungal diversity. A few recent studies on orchid endophytes include those of Oliveira et al. (2014), Yu et al. (2015). , The comprehensive review of Ma et al. (2015) also states that there are very few studies on orchid endophytes from India; these include those of 4 orchids from Kolli hills, Tamilnadu, southern India (Kasmir et al. 2011), 2 orchids from Kaiga forest, Western Ghats, southern India (Sudheep & Sridhar 2012), 3 orchids from Simlipal Biosphere Reserve, eastern India (Behera et al. 2013) and 2 orchids from Western Ghats, Karnataka, southern India (Sawmya et al. 2013). Hence, we studied the diversity of endophytes in 11 orchid species from a hitherto unstudied region *viz*. Arunachal Pradesh, the north eastern most state of India.

Materials & Methods

Collection site

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Healthy and mature leaves and roots of eleven orchid species – *Aerides odorata* (AO), *Arundina graminifolia* (AG), *Cymbidium aloifolium* (CA), *Cymbidium munronianum* (CM), *Dendrobium fimbriatum* (DF), *Dendrobium moschatum* (DM), *Eria flava* (EF), *Paphiopedilum fairrieanum* (PF), *Pholidota imbricata* (PI), *Rhynchostylis retusa* (RR) and *Vanilla planifolia* (VP) were collected from the greenhouses and natural orchidarium of Tipi Orchid Research Centre, West Kameng District of Arunachal Pradesh (27.3428° N, 92.3024° E). The samples were processed within 24h of collection.

Surface sterilization of leaf and root tissues

The leaves were washed thoroughly in running tap water and for each species, one hundred tissue segments (0.5 cm^2) were excised and surface sterilized following the protocol of Suryanarayanan et al. (1998). It consisted of immersing the tissue segments in 70% ethanol of 5 seconds, followed by treatment with sodium hypochlorite (4% available chlorine) for 90 seconds and finally rinsing them in sterile distilled water for 10 seconds. Such tissue segments were plated in Petri dishes (9 cm dia.) containing antibiotic-amended Potato Dextrose Agar (PDA) medium (20 ml).

The root samples were collected from the same individual orchids that were sampled for leaf endophytes and were surface sterilized following the method of Fisher et al. (1993). In this method, the 100 segments (0.5 cm^2) from each species were serially immersed in 75% ethanol of 60 seconds, sodium hypochlorite (4% chlorine) for 180 seconds and in 75% ethanol of 30 seconds. Surface sterilized segments were plated on antibiotic containing PDA as mentioned above.

Incubation and isolation of orchid endophytes

Petri dishes, each with ten leaf or root segments were incubated in a light chamber (12h light: 12 h dark cycle, 2200 lux of light) at 26°C for 4 weeks (Suryanarayanan 1992). The efficacy of the surface sterilization procedure was confirmed by the method of Schulz et al. (1998). The surface sterilized segments were pressed on PDA contained in Petri dishes and removed. These dishes were incubated and periodically observed for the growth of fungi. Absence of any fungal growth in these confirmed the adequacy of the methods.

The endophytes which emerged from the tissue segments were isolated, cultured on PDA slants and identified using standard manuals (Barnett & Hunter 1972, Ellis 1971, 1976, Ellis & Ellis 1988, Sutton 1980, Onions et al. 1981). The sterile isolates were given codes based on culture characteristics such as growth rate, colony surface texture and hyphal pigmentation (Suryanarayanan et al. 1998) and were assumed to represent different taxonomic species (Bills & Polishook 1994).

Statistical analysis

The colonization frequency was calculated by the method of Hata & Futai (1995).

$$CF\ \% = \frac{\text{Number of segments colonized by each endophyte}}{\text{Total number of segments observed}} \times 100$$

Fisher's α was used to estimate species diversity of endophytes. Biodiversity Pro software was used to perform and Correspondence analysis (available from The National History Museum and The Scottish Association for Marine Science – biodiversity@nhm.ac.uk) (McAleece et al. 1997).

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Results

One thousand nine hundred and forty three isolates of endophytes were isolated from the eleven orchid species. Of these, 1024 isolates were recovered from leaf tissue segments and 919 isolates were obtained from root tissues. Overall, 647 isolates belonged to the genus *Xylaria* (Tables 1 & 2).

The CF% of foliar endophytes ranged from 30 in *V. planifolia* to 126 in *A. graminifolia* (Table 1). The leaf of *C. aloifolium* supported the maximum number of endophyte species while those of *A. graminifolia* and *D. moschatum* harboured the minimum number of endophyte species.

Table 1 Species diversity and Colonization Frequency % (CF%) of endophytes isolated from leaves of eleven orchid species.

Fungi	AO	AG	CA	CM	DF	DM	EF	PF	PI	RR	VP	Total
Aspergillus sp.									1			1
Cladosporium sp.	3		2				1	32	2	47	15	102
Colletotrichum sp.	39	75	51	67	53	44	4		37			370
Dendryphion sp.				1								1
Fusarium sp. 1	1		4	4	9	1	2	4		1		26
Fusarium sp. 2			1								1	2
Glomerella sp.										5		5
Lasiodiplodia theobromae			1				1	1				3
Mucor sp.				3								3
Nigrospora sp.			1									1
Paecilomyces sp.			1									1
Penicillium sp.			2					5		2	1	10
Pestalotiopsis sp.		1										1
Phomopsis sp.	1		4	1	1		6		1			14
Phyllosticta capitalensis	11	37	9	9	20	41	69		23	1	2	222
Sterile form sp. 1	2						3				1	6
Sterile form sp. 2								1				1
Sterile form sp. 3					1				1			2
Sterile form sp. 5	3						8	14	5		3	33
Sterile form sp. 6								1				1
Torulomyces sp.										7		7
Trichoderma sp. 1			1									1
Xylaria sp. 1	12	13	26	17	16	23	18		3	14	4	146
Xylaria sp. 2									15			15
Xylaria sp. 3	3		1		3			6	7	8	3	31
Yeast form 1				15	4							19
Total CF%	75	126	104	117	107	109	112	64	95	85	30	1024
Total No. of species	9	4	13	8	8	4	9	8	10	8	8	
Fisher's Alpha	2.67	0.79	3.92	1.94	2.00	0.82	2.31	2.41	2.82	2.16	3.57	

The species diversity was highest for *C. aloifolium* and lowest for *A. graminifolia*. *Xylaria* spp. were present in the leaves of all the orchid species screened; *P. capitalensis* was also wide spread and was present in 10 of the 11 orchids studied. A *Colletotrichum* species was present in 8 species of orchids and was dominant in 7 of them; 370 isolates of the total of 1024 isolates belonged to this species (Table 1).

With respect to root endophytes, the CF% ranged from 37 in *P. fairrieanum* to 125 in *P. imbricata* (Table 2). The maximum number of endophyte species was present in the root of *E. flava* while the minimum number was supported by the root of *R. retusa.* The species diversity of endophytes was highest for *C. munronianum* and lowest for *R. retusa. Xylaria* spp were present in roots of all the orchid species screened. *Xylaria* sp. 1 dominated the endophyte assemblage of 7 orchids; of the total 919 root endophyte isolates, 416 belonged to this species. A *Phomopsis* sp. was also wide spread (total of 272 isolates) and was present in 8 orchid species. A correspondence analysis was performed to determine the overlap between the leaf and root endophyte assemblages. This segregated clearly the endophyte assemblages of the roots from those of the leaves (Fig. 1).

Table 2 Species diversity and Colonization Frequency % (CF%) of endophytes isolated from roots of eleven orchid species.

Fungi	AO	AG	CA	CM	DF	DM	EF	PF	PI	RR	VP	Total
Alternaria sp.			1									1
Arthrinium sp.					1							1
Aspergillus sp.	3											3
Colletotrichum sp.			24	4	5	8						41
Curvularia sp.	1		1									3
Fusarium sp. 1	1	3		1	12	2	5					24
Fusarium sp. 2			3	12			15	13	17	2		62
Fusarium sp. 3								3				3
Lasiodiplodia theobromae						1	5		2		3	11
Paecilomyces sp.									1			1
Penicillium sp.								3				3
Pestalotiopsis sp.	9						4				1	14
Phomopsis sp.	24		14		7	55	54		71	35	13	272
Sterile form sp. 2				1		1			2			4
Sterile form sp. 4		2										2
Sterile form sp. 5		2										2
Sterile form sp. 6			1									1
Torulomyces sp.		1										1
Trichocladium sp. 1							3					3
Trichoderma sp. 1				8	1		1					10
Trichoderma sp. 2							1					1
Xylaria sp. 1	53	52	61	6	30	40	23	15	32	55	50	416
Xylaria sp. 2				2								2
Xylaria sp. 3	11	5		5	14			2				37
Total CF%	101	65	107	39	70	107	110	37	125	92	66	919
Total No. of species	7	6	7	8	7	6	9	5	6	3	4	
Fisher's Alpha	1.71	1.61	1.68	3.05	1.94	1.37	2.32	1.56	1.31	0.59	0.94	

Discussion

Generally, the diversity of endophytes was slightly higher in the leaves that in the roots. This is similar to earlier reports on orchid endophytes (Chen et al. 2011, Sudheep & Sridhar 2012). Although a few endophytes were shared by root and leaf tissues, a correspondence analysis revealed that the there was little overlap between the endophyte assemblages of these two tissues (Fig. 1). This suggests that the endophytes exhibit more tissue specificity than host specificity (Sawmya et al. 2013). In the present study, since all the samples were collected from the same location, it could be assumed that the type of host tissue rather than the environment plays a key role in the distribution of endophytes among the orchids. As early as in 1992, Petrini et al. stated that plant organs constitute specific microhabitats with reference to endophyte colonization. Suryanarayanan & Vijaykrishna (2001) had demonstrated that endophytes exhibit host tissue selection since the endophyte assemblage of the aerial roots and leaves of *Ficus benghalensis* which shared the same environment differed significantly. A few other studies also support this observation (Kumar & Hyde 2004, Qi et al. 2012, Behie et al. 2015). Furthermore, tissue specificity of non-mycorrhizal endophytes has been reported for the orchid *Bletilla ochracea* by Tao et al. (2008). Our present study using eleven different orchid species confirms that endophyte assemblage is determined more by the nature of the plant host tissue. It is possible that the difference between the root and leaf in their habitat and physiology determines their endophyte composition.

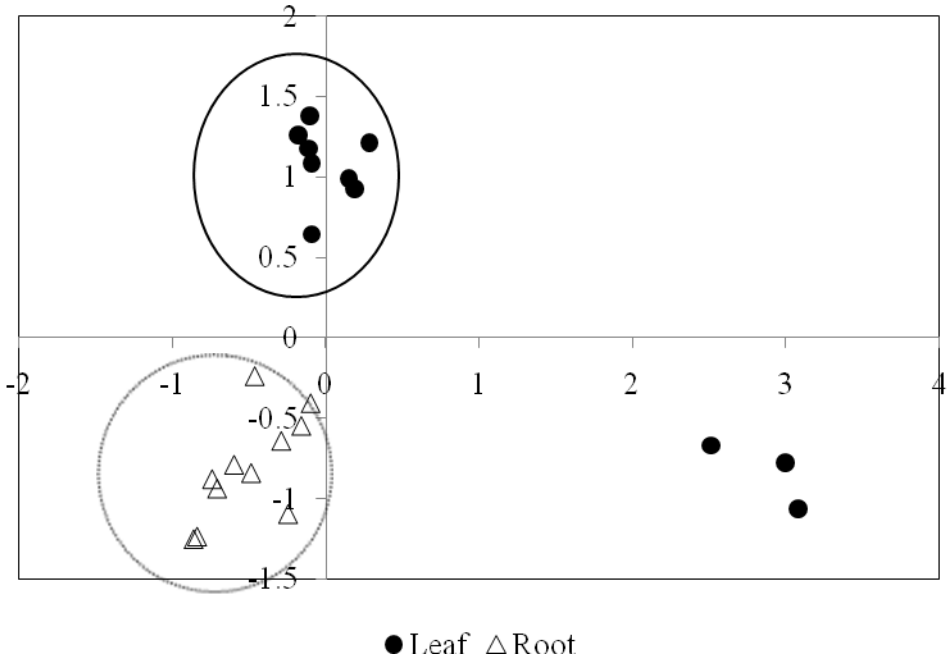

Fig. 1 – Correspondence analysis for endophyte assemblages in leaves and root of orchids.

Xylaria spp. was dominant in the roots of eight of the eleven orchids we studied. They were consistently present in the leaf and roots of orchids screened. Bayman et al. (1997) reported the ubiquitous occurrence of Xylariaceae members in different species of the orchid genus *Lepanthes* growing in Puerto Rico. Chen et al. (2013) found that in seven of *Dendrobium* species screened, Xylariaceous fungi was the most dominant endophyte followed by endophytes belonging to genera such as *Fusarium*, *Colletotrichum* and *Phomopsis*. There are several reports attesting to the wide host range of Xylariaceous fungi as endophytes (Bayman et al. 1998, Okane et al. 2008). Govinda Rajulu et al. (2013) recovered *Xylaria* spp. as foliar endophytes from 22 tree species of 13 different families of a dry thorn forest and 27 tree species of 15 families from a stunted montane evergreen forest in southern India. As endophytes, *Xylaria* spp. exhibit a wide geographic range (Davis et al. 2003). Xylariaceous endophytes are known to survive as saprotrophs in plant litter, elaborate plant cell wall degrading enzymes for litter substrate utilization and produce antimicrobial compounds including antifungal metabolites which could aid in effective competition (Oliveira et al. 2011, Govinda Rajulu et al. 2013). Ratnaweera et al. (2014) reported that a *Xylaria* endophyte of the orchid *Anoectochilus setaceus* produces a nortriterpenoid antibacterial compound. Accumulation of such traits could aid in the niche expansion of Xylariaceous fungi such that they could exist as endophytes in both root and leaf tissues as observed here.

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