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Diversity of endophytic fungi associated with *Hedychium spicatum* Ham ex Sm. and their antifungal activity against the phytopathogen *Alternaria solani*

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Abstract

Twenty eight strains of endophytic fungi including sterile mycelia were isolated from the leaf, rhizome and roots of *Hedychium spicatum* Ham ex. Sm. Majority of the endophytic fungi isolated belonged to the phylum Ascomycota which accounted of about 84%. The endophytic assemblage was dominated by the class Sordariomycetes. Endophytic fungal genera such as *Fusarium* and *Penicillium* were found to be common to all the plant parts. Diversity of endophytic fungi was found to be highest in the roots (H'= 2.43). *Mucor hiemalis*, *Penicillium* sp., *Penicillium expansum*, *P. simplicissimum*, *Phoma medicaginis*, *Trichoderma* sp. and *T. gamsii* showed above 60% inhibition against the phytopathogen *Alternaria solani*.

Key words – Colonization frequency– diversity index – percentage inhibition

Introduction

The kingdom fungi plays important role in the ecosystem. It includes about 1.5 million species and a vast majority are still to be identified (Hawksworth 2001). The kingdom includes parasites, saprophytes and mutualists. The relationship between plant and fungi is a complex and ancient one, however fewer than 10% of fungi are known to colonize plants (Knogge 1996). Plant pathogenic fungi represent relatively a small group of those fungi that are associated with the living plants. Majority of the fungi are saprophytes that depend on dead organic matters for nutrition and others are symbionts and as cryptic colonizers of plants known as 'Endophytes'.

The term endophyte was coined by De Bary in 1866. This term is referred to a wide range of organisms residing within the plant. Fungal endophytes are reported to be present in almost all the plants (Larran et al. 2002). They are known to provide resistance to the host against many phytopathogens and provide stress tolerance (Azevedo et al. 2000, Sturz& Nowak 2000).Vogl (1898) first isolated and cultured asymptomless endophytes from seeds of *Lolium temulentum*. Based on their nature, endophytic fungi are categorized into three groups viz. pathogens of another host plant that do not cause infections in other plants in which they reside, microbes which are non pathogenic and pathogens that have been rendered nonpathogenic but are still capable of

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colonization by selection methods or genetic alteration (Backman & Sikora 2008). Most fungal endophytes isolated to date have been identified as ascomycetes and their asexual morphs; however Rungjindamai et al. (2008) showed that several endophytic fungi may also be basidiomycetes. Endophytic fungi have been found in most plant organs; they can enter the plant through the roots and remain there or become systemic and colonize stem, leaves and even flowers and fruits. However, roots are not the only way of entrance; many phyllosphere endophytes enter the plant trough stomata or wounds (McCully 2001, Schulz & Boyle 2006, Danhorn & Fuqua 2007). While some endophytic fungi appear to be ubiquitous (e.g. *Fusarium* spp., *Alternaria* spp., *Pestalotiopsis* spp., *Aspergillus* spp, *Botryosphaeria* spp.) others apparently present host specificity and/or host preference (Petrini 1996, Suryanarayanan & Kumar 2000, Schulz & Boyle 2006, Hu et al. 2007, Slippers & Wingfield 2007, Pang et al. 2008, Toju et al. 2013).

The present work was conducted to study the endophytic fungal diversity of *Hedychium spicatum* Ham ex Sm and antifungal activity of the selected endophytic fungi against the pathogen *Alternaria solani*.

Alternaria solani is known to cause various diseases in the commercially important plant tomato such as early blight (foliage), collar rot (basal stem of seedlings), stem lesions (stem of adult plants) and fruit rot (fruits). Early blight is the most destructive of all of the other diseases.

The plant *Hedychium spicatum* belongs to the family Zingiberaceae. It is found from Himachal Pradesh to Arunachal Pradesh, at altitudes of 1800-2800 m. It grows to around 1–1.5 m, with leafy stems. Flowers are fragrant, white with an orange-red base, appearing in a dense spike, 15–25 cm, at the top of the stem. The rootstock is carminative, expectorant, stimulant, stomachic and tonic. It is useful in the treatment of liver complaints, and is also used in treating fevers, vomiting, diarrhea, inflammation, pains and snake bite. The aim of this study is to identify the fungal endophytes using culture dependent techniques and to study the antifungal activity of selected endophytes against the phytopathogen *A. solani*.

Materials & Methods

Study site and collection of plant material

The plant materials of *Hedychium spicatum* were collected from Mawlai, East Khasi Hills, The study site Mawlai is situated at an altitude of 1400 msl and lies at 25^{o} 36' 41.2" N latitude and 91^{o} 53' 59.2" E longitude.

Culture, identification and isolation

Leaf, stem and roots were collected aseptically from the plant *Hedychium spicatum* and were processed within 24 hours of collection following the methods of Suryanarayanan et al. (2003).

Potato dextrose agar medium (PDA) was used for the isolation and identification of endophytic fungi. The samples were thoroughly washed in tap water and were cut into small pieces (5mm for leaf, 1cm for stem and root). To eliminate the epiphytic microorganisms all the samples were surface sterilized by immersing in 70% alcohol for 1–3 minutes, followed by Sodium hypochlorite (4% available chlorine) for 3–5 minutes and again washed in alcohol for 2–5 seconds before a final rinse in distilled water. The samples were dried in laminar airflow before placing them in the Petri-dish containing PDA medium amended with streptomycin sulphate (100 mg/L). The Petri-dishes were incubated for 7–10 days in incubator at 25±2^{o}C. Hyphae from the emerging colonies were again subcultured in Czapek Dox Agar media for 5–7 days to obtain pure culture. Identification of the isolated endophytic fungi was carried out based on their morphological and reproductive structures using standard manuals (Ellis 1976, Domsch et al. 1980, Barnett & Hunter 1998) and websites, www.indexfungorum.com and www.mycobank.com.

Data analysis

The colonization frequency (CF %) of each endophytic fungi was calculated following the method of Hata & Futai (1995).

$$CF\ (\%) = (N_{col}/N_t) \times 100$$

Where:
N_{col} = the number of segments colonized by each endophytic fungi
N_t = the total number of segments observed
Shannon- Wiener diversity index and Simpsons's index was calculated by the formulae:
Shannon- Wiener diversity index= $-\Sigma[(pi) \times \ln(pi)]$
Simpson's dominance index=$\Sigma(pi)^2$
Where pi= (n/N), pi= proportion of colonization of the *i*th species in a sample.
Jaccard's index (JI %) = c/(a+b-c) x 100
a= No. of species in community 1
b= No. of species in community 2
c= No. of species common between the two assemblages.
Shannon's Diversity and Simpsons dominance indices were calculated using the software PAST

Antimicrobial activity

Antimicrobial activity of the endophytic fungi against the phytopathogen *Alternaria solani* was studied following the method of Skidmore & Dickinson (1976). Fourteen endophytic fungi were selected to test their antimicrobial activity against the phytopathogen *Alternaria solani*. Discs of pathogen and the antagonist (5 mm diameter each) were placed diametrically opposite to each other on the Petri dish containing PDA medium. The plates were incubated at 25±2˚ C for 7–9 days Three replicates were maintained and pure cultures of the pathogen and endophytic fungi were maintained as controls. The growth of antagonist and pathogen colonies were measured after 5 days of incubation at regular intervals of 24 hours till the 8th day. The percentage inhibition of the pathogen was calculated using the following equation based on Keshavachandran et al. (2007).

$$\%\ \text{inhibition} = \frac{Y-Z}{Y} \times 100$$

Where:
Y= mycelial growth of the pathogen in pure culture (control)
Z= mycelial growth of the pathogen along with the antagonist

Results

Diversity

A total of 28 strains of endophytic fungi including sterile mycelia were isolated from the leaf, rhizome and root of *Hedychium spicatum*. Highest number of species were isolated from the root (16 species) followed by the leaf (15 species) and the stem (13 species). However not much difference was observed between the numbers of species isolated from each part of the plant. 84.00% of the isolated endophytic fungi belonged to Ascomycota followed by Zygomycota and Oomycota which accounted for 8.00% each (Fig. 1).The isolated fungi belonged to the classes Dothideomycetes, Eurotiomycetes, Mucoromycetes, Saccharomycetes and Sordariomycetes (Table 1). Sordariomycetes was found to be the predominant class in the endophytic assemblage of the plant (Fig. 2). Endophytic fungi such as *Fusarium* sp.1, *Fusarium* sp.2 and *Penicillium simplicissimum* were found to be common to all the plant parts. *Juxtiphoma eupyrena* was found to be dominant in the leaves with colonization frequency of 12.50%. *Fusarium* sp.1 and *Fusarium* sp.2 were found to be dominant in the rhizome with colonization frequency of 7.64% and in the roots highest dominance was exhibited by *Fusarium* sp.2 with colonization frequency of 13.89% (Table 2).

The genera specific to the leaves were *Acremonium, Alternaria, Arthrinium, Cladosporium, Colletotrichum, Globisporangium* and *Pyhtium*. Four genera were found to be specific to the

rhizomes which were *Absidia, Ilyonectria, Mucor* and *Trichoderma*. *Humicola, Meyerozyma* and *Nectria* were present in the underground parts such as rhizome and roots. (Table 2).

The diversity indices of the endophytic assemblage of *H. spicatum* are shown in Table 3. Higher the values of Shannon's diversity index (1.5–3.5) and closer the Simpson's diversity index to 1 the more diversified is the microbial assemblage. Diversity of endophytic fungi was found to be highest in the roots (H'= 2.43) followed by the rhizome (H'= 2.42) and the leaf (H= 2.41). Similarly Simpson's diversity index (0.88–0.89) was found to be almost same in all the tissues which show a great degree of diversity of endophytic community in all the tissues. Simpson's Dominance Index (D) ranged between 0.11–0.12 which indicates that the individuals belonged to diverse genera and species. Jaccard's index showed that the species similarity of the endophytic community of the leaf tissue overlapped the rhizome tissue by 15.15%, species composition of rhizome tissue overlapped the root tissue by 23.68% and the species composition of the leaf overlapped the root by 18.42%.

Antifungal activity

Fourteen endophytic fungi were tested for their antifungal activities. Among them 10 strains showed more than 50% inhibition towards the phytopathogen *A. solani*. It was observed that *Trichoderma* sp. grew faster than other isolates in PDA and showed maximum inhibition towards *A. solani* with 66.30%. Overall the ranks *Trichoderma* and *Penicillium* showed inhibition of 60% and above. Apart from these ranks the other endophytes which showed similar percentage inhibitions were *Mucor hiemalis* and *Phoma medicaginis*. *Absidia* sp, *Acremonium cerealis*, *Fusarium* sp. 1 and *Meyerozyma caribbica* inhibited the pathogen by 50–59%. The least inhibition was shown by *Juxtiphoma eupyrena* with approximately 26.67% (Table 4).

Discussion

The present work reported the isolation and identification of cultureable endophytic fungi from different plant tissues and the antimicrobial activity of the selected endophytic fungi against the pathogen *A. solani* using Potato Dextrose Agar media (PDA).

Majority of the isolated strains belonged to the phylum Ascomycota. Ascomycota was found to be the most dominant phylum in all the plant parts and also accounted about 84.00% of the assemblage which is in accordance to the findings of Park et al. (2017). Sordariomycetes was the predominant class with 40% of the endophytic fungi as shown from the findings of Li et al. (2016). The genera *Fusarium* and *Penicillium* were common to all the plant parts however, the genus *Fusarium* was found to be more dominating in the rhizome and the roots (Imazaki & Kadota 2015, Potshangbam 2017). Species of *Fusarium* have also been isolated from the healthy rhizomes of *Dioscorea zingiberensis* (Zhang et al. 2010). Some isolated endophytic fungi showed specificity for particular tissues types. This was also evident from previous studies which showed specificity of endophytic fungi for particular tissue types (Errasti et al. 2010, Sánchez-Márquez et al. 2010, Wearn et al. 2012). The genus *Acremonium* which has been found specific to the leaves is also reported as a major grass endophyte (White 1987), it also has a wide host range among non grass plants which includes *Vitis vinifera* (Arnone et al. 2009), *Rhizophora apiculata*(Rukachaisirikul et al. 2012), *Actinidia macrosperma* (Lu et al. 2012) and *Bursera simaruba* (Gonzalez et al. 2016).*Humicola* sp. and *Ilyonectria* sp. have also been isolated from *Fragaria vesca* (Yokoya et al. 2017) and the specificity of *Humicola* to the roots has also been reported by the same authors. The endophytes such as *Juxtiphoma eupyrena* isolated from the plant are known to be pathogenic to several plant species. It was observed that Shannon's diversity index was higher in the rhizome and roots as compared to the leaves. Previous studies have also reported similar results (Rivera-Oduna et al. 2011, Jin et al. 2013). The greater diversity of endophytic fungi in the underground parts specifically the rhizome and roots is due to the presence of soil borne fungi which are more diversified than the air borne fungi which colonize the aerial parts (Jin et al. 2013).

Fourteen endophytic fungi were tested for their antimicrobial activities against the pathogen *Alternaria solani* out of which maximum inhibition percentage was shown by the ranks

Trichoderma and *Penicillium*. The genus *Trichoderma* is also a well known biocontrol agent with outstanding capability to interact with plant and plant pathogens (Spiegel & Chet 1998, Mukherjee et al. 2012) has the ability to efficiently compete for space and nutrient with the target pathogen thus depriving the pathogen of its basic requirements (Siameto et al. 2010). The efficacy of *T. gamsii* in controlling growth of phytopathogens in vitro have been reported by Chen et al. (2016) which is also attributed to its efficiency in competing with the target pathogen. The mycoparasitism process of Trichoderma sp. involves the lysis of protoplasm of hyphae, endoconidia and chlamydospores as observed by Ramanujam et al. (2002) in case of *T. harzianum* and *T. viride* against *Thielaviopsis paradoxa. Trichoderma* sp also produce volatile organic compounds such as alcohols, Ketones, alkanes, furanes, pyrones and terpenes which have varying degree of antagonistic activity against pathogens (Stoppacher et al. 2010). Members of the genus *Penicillium* have also shown good inhibitory effect in vitro which may be due to production of the secondary metabolite β-1-3- glucanase, a cell lytic enzyme which inhibits the growth of pathogen (Druvefors et al. 2002). Higher percentage of inhibition was also shown by the strains belonging to the genera of *Acremonium*, *Absidia*, *Fusarium* and *Meyerozyma*. *Acremonium* sp. isolated as an endophyte from *Zingiber officinale* have been found to be an antagonist against soft rot pathogen *Pythium myriotylum* (Anisha & Radhakrishnan 2015).*Absidia* sp. is also known to show antagonistic activity against the rice pathogen *Magnaporthe grisea* (Atugala & Deshappriya 2015). Certain species of *Fusarium* is known to control the root rot disease in *Pisum sativum* and *Meyerozyma caribbica* is known to possess antifungal activity against *Colletotrichum gloeosporioides* (Sisic et al. 2017, Bautista-Rosales et al. 2013). Certain species of *Fusarium* are also known to suppress several soil borne pathogen (Macia-Vicente et al. 2008) and*Alternaria solani* is one of them (Chaerani & Voorrips 2006) which is the reason it may show inhibitiory activity in vitro. The endophytic fungi showing high levels of inhibitory activity against *A. solani* could be used as potential biocontrol agents against diseases caused by the phytopathogen. Further studies are required to throw light into other prospects of these endophytes.

Table 1 List of endophytic fungal strains isolated from the tissues of *Hedychium spicatum*

Sl. No.	**Endophytes**	**Family**	**Order**	**Class**	**Phylum**
1	*Globisporangium intermedium*	Pythiaceae	Peronosporales	Incertae sedis	Oomycota
2	*Pyhtium aphanidermatum*				
3	*Absidia* sp.	Cunninghamellaceae	Mucorales	Mucoromycetes	Zygomycota
4	*Mucor hiemalis*	Mucoraceae			
5	*Cladosporium cladosporioides*	Cladosporiaceae	Capnodiales	Dothideomycetes	Ascomycota
6	*C. macrocarpum*				
7	*Alternaria alternata*	Pleosporaceae	Pleosporales		
8	*Juxtiphom a eupyrena*	Didymellaceae			
9	*Phoma medicaginis*				
10	*Penicillium* sp.				
11	*P. expansum*				
12	*P. simplicissimum*				
13	*P. spinulosum*				
14	*Meyerozyma caribbica*	Debaryomycetaceae	Saccharomycetales	Saccharomycetes	
15	*Colletotrichum* sp.	Glomerallaceae	Glomerellales	Sordariomycetes	
16	*Acremonium cerealis*	Incertae sedis	Hypocreales		
17	*Ilyonectria* sp.				
18	*Fusarium* sp.1	Nectriaceae			
19	*Fusarium* sp.2				
20	*F. redolens*				
21	*Nectria inventa*				
22	*Trichoderma* sp.	Hypocreaceae			
23	*T. gamsii*				

Table 1 Continued.

Sl. No.	Endophytes	Family	Order	Class	Phylum
24	*Humicola fuscoatra*	Chaetomiaceae	Sordariales		
25	*Arthrinium arundinis*	Apiosporaceae	Incertae sedis		

Table 2 Percentage colonization frequency of endophytic fungal strains from different tissues of *H. spicatum*

Sl. No.	Endophytes	CF (%)		
		Leaf	Rhizome	Root
1	*Absidia* sp.	-	0.69	-
2	*Acremonium cerealis*	1.39	-	-
3	*Alternaria alternata*	1.39	-	-
4	*Arthrinium arundinis*	2.78	-	-
5	*Cladosporium cladosporioides*	2.78	-	-
6	*C. macrocarpum*	1.39	-	-
7	*Colletotrichum* sp.	1.39	-	-
8	*Fusarium* sp.1	4.17	7.64	2.08
9	*Fusarium* sp.2	1.39	7.64	13.89
10	*F. redolens*	-	-	0.69
11	*Globisporangium intermedium*	2.78	-	-
12	*Humicola fuscoatra*	-	-	3.47
13	*Ilyonectria* sp.	-	2.78	-
14	*Juxtiphoma eupyrena*	12.50	-	3.47
15	*Meyerozyma caribbica*	-	-	1.39
16	*Mucor hiemalis*	-	2.08	-
17	*Nectria inventa*	-	-	0.69
18	*Penicillium* sp.	-	2.78	1.39
19	*P. expansum*	-	0.69	1.39
20	*P. simplicissimum*	1.39	3.47	4.17
21	*P. spinulosum*	-	-	0.69
22	*Phoma medicaginis*	5.56	-	2.78
23	*Pythium aphanidermatum*	1.39	-	-
24	*Trichoderma gamsii*	-	4.86	3.47
25	*Trichoderma* sp.	-	6.25	-
26	Mycelia sterilia (Black)	8.33	2.08	3.47
27	Mycelia sterilia (White)	4.17	2.08	4.17
28	Mycelia sterilia (Yellow)	-	6.94	2.08

'-'= absent

Table 3 Diversity indices of endophytic fungi from the tissues of *H. spicatum*

Diversity indices	Leaf	Rhizome	Root
Shannon Weiner's index (H)	2.41	2.42	2.43
Simpson's Dominance index (D)	0.12	0.11	0.12
Simpson's Diversity index (1-D)	0.88	0.89	0.88

Table 4 Antifungal activity of selected endophytic fungal strains against the pathogen *Alternaria solani*

Sl. No.	Endophytes	Pathogen		
		Growth in control (mm)	Growth with antagonist (mm)	Percentageinhibition (%)
1	*Absidia* sp.	90	37.33±3.71	58.52±4.12
2	*Acremonium cerealis*	70	33.33±2.91	52.38±4.15

Table 4 Continued.

Sl. No.	Endophytes	Pathogen		
		Growth in control (mm)	Growth with antagonist (mm)	Percentage inhibition (%)
3	*Cladosporium cladosporioides*	70	36.67±0.33	47.62±0.48
4	*Fusarium* sp. 1	70	29.33±0.67	58.10±0.95
5	*Fusarium* sp. 2	90	49.33±0.33	45.19±0.37
6	*Juxtiphoma eupyrena*	70	51.33±1.86	26.67±2.65
7	*Meyerozyma caribbica*	70	34.33±0.33	50.95±0.48
8	*Mucor hiemalis*	75	28.33±1.67	62.22±2.22
9	*Penicillium* sp	50	33.33±4.41	33.33±8.82
10	*P. expansum*	50	18.33±1.67	63.33±3.33
11	*P. simplicissimum*	75	29.33±5.17	60.89±6.90
12	*Phoma medicaginis*	70	26.00±2.08	62.86±2.97
13	*Trichoderma gamsii*	80	29.33±2.33	63.33±2.92
14	*Trichoderma* sp.	90	30.33±0.88	66.30±0.98

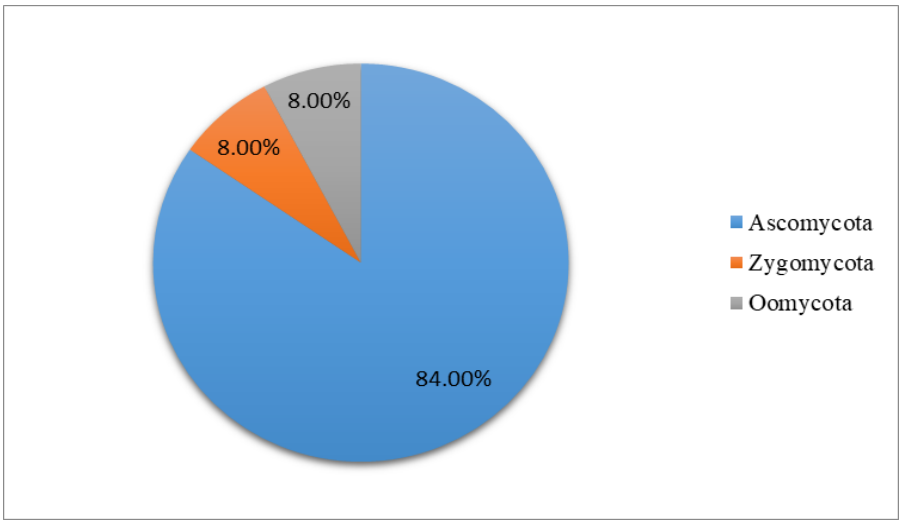

Fig. 1 – Percentage of occurrence of endophytic fungi from different phyla

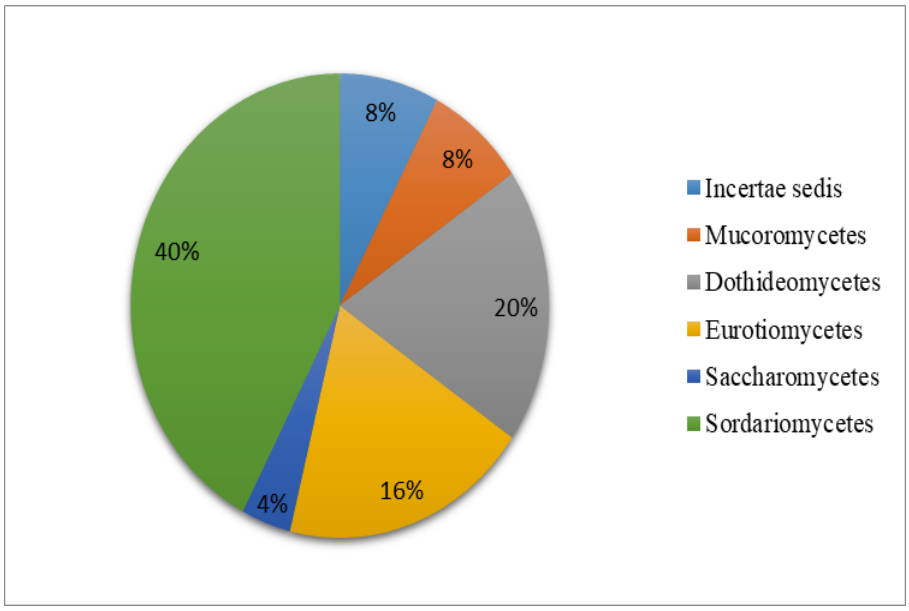

Fig. 2 – Percentage of occurence of endophytic fungi from different classes

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