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Research Article

Diversity of Foliar Endophytic Fungi in *Artocarpus heterophyllus* Lam. and *Citrus reticulata* Blanco of Tripura

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ABSTRACT

Present investigation dealt with the isolation and diversity of foliar fungal endophytes from two fruit yielding plants of Tripura state. In addition, seasonal distributions of endophytes regarding fruiting and non-fruiting seasons in two host plants were also studied. Twenty one fungal strains along with one nonsporulating hyaline form were isolated as foliar endophytes from the two host plants. In both fruiting and non-fruiting seasons from *Citrus reticulata*, seven foliar endophytic fungi were isolated. Meanwhile, from *Artocarpus heterophyllus* in both the seasons, eleven fungal endophytes with one nonsporulating hyaline form were also isolated. The relative frequencies of the isolated fungi from both *A. heterophyllus* and *C. reticulata* in fruiting and non-fruiting season significantly differed among the sampling sites, whereas relative frequencies individual endophytic fungus showed no significant differences among various sampling sites.

Keywords: Microfungi, Artocarpus heterophyllus, Citrus reticulata, Fruiting and non-fruiting season, Fungal diversity

Introduction

Endophytes are microorganisms, usually, fungi and bacteria that live within the plant tissues intercellularly and intracellularly for a period of their life cycle without producing any visible symptoms of the disease under normal conditions [1]. Fungal endophytes can invade and live inside the tissues of the living host plant and do not cause any visible injury to the host plant [2]. They inhabit the interior of leaves, branches, stems of plants, without any apparent damage to the host plant [3]. Relationship between fungal endophyte and the host plants range from mutualistic or symbiotic to antagonistic or slightly pathogenic [4]. According to Hawksworth and Lücking [5], 3 to 8% of the estimated fungal diversity is known. Endophytic mycoflora can be regarded as an important component of fungal biodiversity of plants. Colonization and distribution of endophytic fungi in host plant mostly determined by the host plant itself [6]. According to Paulus *et al.* [7] variation in chemical profiles of the host plant influence the differential distribution of endophyte assemblages in different hosts. Endophytic fungi reported to have the ability to produce bioactive secondary metabolites with activities identical or almost same of their respective hosts [8]. *Artocarpus heterophyllus* is a popular fruit in different parts of India for easy availability in summer seasons. The plant belongs to the Moraceae family and reported to be a rich source of antidiabetic, antimicrobial, antioxidant and antibacterial agents [9]. The plant was also reported to be effective in treating diarrhoea, fever, dermatitis and cough. The methanolic extracts of leaves possess broad-spectrum antibac-[terial properties against different gram-positive and gram-negative bacteria [10]. Not only that, methonlic extracts of leaves also exhibited an inhibitory effect on various cariogenic bacteria [11]. Ash of jack fruit leaves reported to have wound healing effects and heal ulcers also. The infusion of mature leaves was effective in treating diabetes,

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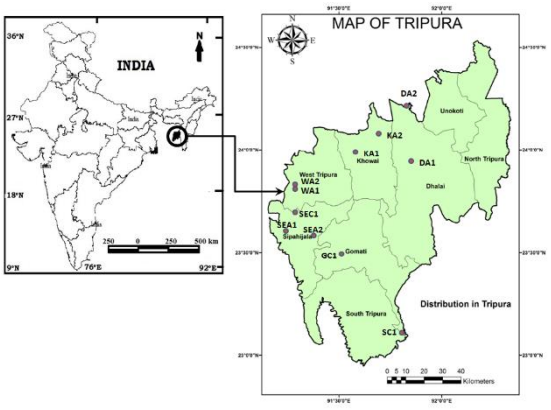

Figure 1. The different sampling sites of *Artocarpus heterophyllus* and *Citrus reticulata* in Tripura

gall stones, and relieve asthma [12]. The medicinal activities of the genus *Citrus* are due to the presence of many medicinally active secondary metabolites such as essential oils, flavonoids etc. [13, 14]. *Citrus reticulata* has many physiological, pharmacological and medici-nal activities such as antimicrobial, antioxidant and antiproliferative activities [15, 16]. The plant was used in many folk medicines to treat such as fever, stomachache, edema, cardiac diseases, bronchitis, and asthma [17]. Two important fruit yielding plants of Tripura state i.e. *A. heterophyllus* and *C. reticulata* with medicinal properties, were selected for the study. Moreover, this was the first attempt to document foliar endophytic fungal communities from the two host plants' of Tripura state.

Material and Methods

Sampling of sites

For a comprehensive sampling of host plants, the zone of occurrence for particular fruit-yielding plants was identified. The sampling of sites in the zone of occurrence was done by stratified random sampling method. For *A. heterophyllus* Lam., sampling of explants was done from four districts (West, Sepahijala, Khowai, and Dhalai- two sites from each District) and for *C. reticulata* Blanco three districts (Sepahijala, Gomati and South- one site from each District) were considered for sampling (Figure 1).

Collection of host plants

Green leaves from healthy and disease-free host plants of Jackfruit (*A. heterophyllus* Lam.) and Orange (*C. reticulata* Blanco) were selected to minimize the presence of pathogenic and Saprobic species and collected from each of the sampling sites in sterile bags and processed within 24 hours of collection. The collection of samples was carried out from December 2016 to July 2018.

Isolation of endophytes

Isolation of fungal endophytes was done according to the standard protocol [18, 19] with slight modification. Exposure to sterilant was decided based on the tissue strength of explants. Sterilization protocol was validated using a leaf imprint method [18]. Four to five segments of plant tissues were placed on Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) plate supplemented with streptomycin (100 µg/ml), and incubated in a BOD incubator for 21 days at 25± 2°C. Hyphal tips from fungus growing out from the samples were subsequently transferred onto fresh PDA and or MEA plates to isolate pure colonies.

Identification of fungi

Identification was made using macroscopic characteristics of colonies and microscopic characteristics. The standard manuals and literature were used for identification of fungi [20 – 24]. For doubtful identities, identification was authenticated (morphological identification) by NFCCI, Agharkar Research Institute, Pune. Nomenclature was authenticated from Index Fungorum (www.indexfungorum.org) and MycoBank (www.mycobank.org) databases.

Data analysis

Colonization rate (CR) was calculated as the total number of plant tissues colonized by endophytes divided by the total number of plant pieces incubated for particular plant samples and expressed as percentage. Isolation rate (IR) is a measure of endophytic richness in a given sample for plant tissues i.e. the incidence of multiple colonizations of endophytes per piece and was calculated as the number of isolates obtained divided by the total number of tissue pieces but not expressed as percentage. Relative frequency (RF) of isolation used to represent fungal density and was calculated as the number of isolates of a species divided by the total number of isolates and expressed also as percentage [25, 26].

Diversity indices

Diversity indices were calculated according to

Table 1. Host wise occurrence of foliar endophytic fungi in fruiting and non-fruiting seasons

Sl. No.	Name of the fungal strains	Fruiting Season		Non-fruiting Season		Family	Order
		Cr	*Ah*	*Cr*	*Ah*		
1	*Alternaria alternata* (Fr.) Keissl.	-	-	-	+	Pleosporaceae	Pleosporales
2	*Aspergillus fumigatus* Fresen.	-	+	-	+	Aspergillaceae	Eurotiales
3	*Aspergillus japonicus* Saito	-	+	+	+	Aspergillaceae	Eurotiales
4	*Cladosporium oxysporum* Berk. & M.A. Curtis	-	+	-	+	Cladosporiaceae	Capnodiales
5	*Colletotrichum artocarpi* Delacr.	-	+	-	-	Glomerellaceae	Glomerellales
6	*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc	+	-	+	-	Glomerellaceae	Glomerellales
7	*Diaporthe rosae* Samarakoon & K.D. Hyde	-	-	-	+	Diaporthaceae	Diaporthales
8	*Epicoccum nigrum* Link	+	-	-	-	Didymellaceae	Pleosporales
9	*Fusarium equiseti* (Corda) Sacc.	-	+	-	+	Nectriaceae	Hypocreales
10	*Fusarium oxysporum* Schltdl.	-	+	-	-	Nectriaceae	Hypocreales
11	*Fusarium incarnatum*	+	-	-	-	Nectriaceae	Hypocreales
12	*Nigrospora oryzae* (Berk. & Broome) Petch	-	+	-	+	Apiosporaceae	Xylariales
13	*Nigrospora sacchari* (Speg.) E.W. Mason	+	-	-	-	Apiosporaceae	Xylariales
14	*Nodulisporium gregarium* (Berk. & M.A. Curtis) J.A. Mey.	+	-	+	-	Hypoxylaceae	Xylariales
15	*Penicillium citrinum* Thom	-	+	+	+	Aspergillaceae	Eurotiales
16	*Penicillium oxalicum* Currie & Thom	+	-	-	-	Aspergillaceae	Eurotiales
17	*Pestalotiopsis neglecta* (Thüm.) Steyaert	-	-	+	-	Pestalotiopsidaceae	Amphisphaeriales
18	*Pestalotiopsis versicolor* (Speg.) Steyaert	-	+	-	+	Pestalotiopsidaceae	Amphisphaeriales
19	*Phomopsis citri* (Sacc.) Traverso & Spessa	-	-	+	-	Diaporthaceae	Diaporthales
20	*Xylaria feejeensis* (Berk.) Fr.	+	+	+	+	Xylariaceae	Xylariales
21	Nonsporulating hyaline form I	-	+	-	+	–	–

Note: Cr: *C. reticulata* and Ah: *A. heterophyllus*

Hammer *et al.* [27] using PAST software.

Statistical analysis

All the assays were performed in triplicate and the results were calculated using Origin version 7.0 and expressed as Mean values. In addition, colonization and relative frequencies of two plants collected from various sites using two-way ANOVA were analyzed, where the collection sites of the plant and fungal isolates were used as factors.

Results and Discussions

Total thirty explants were inoculated from each sampling site in both fruiting and non-fruiting seasons. Thus ninety explants for *C. reticulata* and two hundred forty explants for *A. heterophyllus* were screened each season. Total twenty one fu-

Table 2. Site wise Colonization Rate and Isolation Rate of foliar endophytic fungi from explants of different host plant

Districts	Site Code	Host Plant	Colonization Rate (CR)		Isolation Rate (IR)	
			Fruiting Season	Non- fruiting Season	Fruiting Season	Non- fruiting Season
Gomati	GC1	*C. reticulata*	80	84	0.88	0.88
South Tripura	SC1		84	86	0.92	0.9
Sepahijala	SEC1		72	80	0.82	0.82
West Tripura	WA1	*A. heterophyllus*	66	66	0.73	0.76
	WA2		70	78	0.8	0.76
Sepahijala	SEA1		66	70	0.76	0.8
	SEA2		73	73	0.73	0.83
Khowai	KA1		60	66	0.66	0.73
	KA2		66	73	0.76	0.88
Dhalai	DA1		60	63	0.7	0.76
	DA2		63	70	0.7	0.73

Note: GC1: Gomati *Citrus* 1, SC1: South *Citrus* 1, SEC1: Sepahijala *Citrus* 1, WA1: West *Artocarpus* 1, WA2: West *Artocarpus* 2, SEA1: Sepahijala *Artocarpus* 1, SEA2: Sepahijala *Artocarpus* 2, KA1: Khowai *Artocarpus* 1, KA2: Khowai *Artocarpus* 2, DA1: Dhalai *Artocarpus* 1, DA2: Dhalai *Artocarpus* 2.

Table 3. Relative frequency of foliar endophytic fungi isolated from *C. reticulata* in fruiting and non-fruiting seasons from different sites

Sl. No.	Name of the fungi	Fruiting Season			Non-fruiting Season		
		GC1	SEC1	SC1	GC1	SEC1	SC1
1	*Aspergillus japonicus*	0	0	0	11.36	17.07	15.55
2	*Colletotrichum gloeosporioides*	22.72	26.82	26.08	11.36	12.29	13.33
3	*Epicoccum nigrum*	15.9	9.75	13.04	0	0	0
4	*Fusarium incarnatum*	11.36	17.07	15.21	0	0	0
5	*Nodulisporium gregarium*	18.18	19.51	15.21	25	24.39	22.22
6	*Nigrospora sacchari*	9.09	7.31	8.69	0	0	0
7	*Penicillium citrinum*	0	0	0	15.9	21.95	17.77
8	*Penicillium oxalicum*	13.63	9.75	10.86	0	0	0
9	*Pestalotiopsis neglecta*	0	0	0	11.36	9.75	11.11
10	*Phomopsis citri*	0	0	0	18.18	9.75	11.11
11	*Xylaria feejeensis*	9.09	9.75	10.86	6.81	4.87	8.88

ngal strains and one nonsporulating hyaline form were isolated as foliar endophytes from the two host plants. In both fruiting and non-fruiting seasons, from *C. reticulata* seven foliar endophytic fungi were isolated. From *A. heterophyllus* in non-fruiting season and fruiting season, eleven fungal endophytes with one nonsporulating hyaline forms were isolated. Host wise distributions of foliar endophytes in fruiting and non-fruiting seasons were represented along with their presence and absence (Table 1).

The highest colonization rate and isolation rate of fungal endophytes in *C. reticulata* were recorded in explants collected from the sampling site of South District in both seasons. In *A. heterophyllus* maximum colonization rate was observed in explants collected from site 2 of Sepahijala District and the explants of site 2 of West District in fruiting season and non-fruiting season respectively. But, the highest isolation rate was observed in the fruiting season for explants of site 2 of West district and explants of site 2 of Khowai District in non-fruiting season (Table 2).

Among isolated fungal endophytes in fruiting season from *C. reticulata*, maximum relative frequency was observed in *C. gloeosporioides* but in non-fruiting season, *Nodulisporium gregarium* showed the highest relative frequency (Table 3).

Table 4. Relative frequency of foliar endophytic fungi isolated from *A. heterophyllus* in fruiting and non-fruiting season from different sites

Sl. No.	Name of the fungi	Fruiting Season							Non-fruiting Season						
		WA1	WA2	SEA1	SEA2	KA1	KA2	DA1	DA2	WA1	WA2	SEA1	SEA2	KA1	KA2	DA1	DA2
1	*Alternaria alternata*	0	0	0	0	0	0	0	0	0	8.69	0	12	0	9.09	0	4
2	*Aspergillus japonicus*	0	0	13.04	0	0	0	0	0	0	0	12.5	0	0	0	18.18	20
3	*Aspergillus fumigatus*	13.63	0	0	0	0	14.28	0	0	17.39	0	0	0	0	13.63	0	12
4	*Cladosporium oxysporum*	0	14.28	0	9.09	9.52	14.28	0	8.69	0	13.04	12.5	12	0	13.63	22.72	0
5	*Colletotrichum artocarpi*	22.72	14.28	0	22.72	0	19.04	0	17.39	0	0	0	0	0	0	0	0
6	*Diaporthe rosae*	0	0	0	0	0	0	0	0	39.14	43.47	25	24	17.39	36.36	36.36	40
7	*Fusarium oxysporum*	13.63	0	13.04	0	0	0	0	21.73	0	0	0	0	0	0	0	0
8	*Fusarium equiseti*	0	0	26.08	13.63	47.61	0	25	0	21.73	26.28	45.83	44	52.17	0	0	16
9	*Nigrospora oryzae*	50	38.09	39.13	45.45	14.28	47.61	50	43.47	0	0	4.16	0	8.69	27.27	0	0
10	*Penicillium citrinum*	0	23.8	0	9.09	0	0	0	0	13.04	0	0	0	13.04	0	13.63	0
11	*Pestalotiopsis versicolor*	0	0	0	0	23.8	0	15	0	8.69	8.69	0	0	0	0	0	0
12	*Xylaria feejeensis*	0	9.52	8.69	0	0	4.76	0	8.69	0	0	0	0	8.69	0	0	8
13	Nonsporulating hyaline form	0	0	0	0	4.76	0	10	0	0	0	0	8	0	0	9.09	0

Note: WA1: West *Artocarpus* 1, WA2: West *Artocarpus* 2, SEA1: Sepahijala *Artocarpus* 1, SEA2: Sepahijala *Artocarpus* 2, KA1: Khowai *Artocarpus* 1, KA2: Khowai *Artocarpus* 2. DA1: Dhalai *Artocarpus* 1. DA2: Dhalai *Artocarpus* 2

The relative frequencies of isolated fungi from *C. reticulata* in fruiting (sites: F = 64.45, $p < 0.001$) and non-fruiting season (sites: F = 45.15, $p < 0.001$) significantly differed among the sampling sites, whereas relative frequencies of individual endophytic fungal strain showed no significant differences among the sampling sites.

In the present study, *Nodulisporium* sp. and *Colletotrichum gloeosporioides* were isolated as foliar endophytes. Moreover, relative frequency of *C. gloeosporioides* was maximum in the fruiting season which correlated with earlier reports where *C. gloeosporioides* was found as a frequently isolated or as dominant foliar endophyte of *C. limon*. Most of the taxa isolated had relative frequencies of > 2%, corresponding to the present findings [28, 29]. *C. gloeosporioides* was also reported as the most frequently isolated fungus from the leaves of *C. paradisi* × *C. reticulata* rootstock [30].

In *A. heterophyllus* maximum relative frequency was observed in *Nigrospora oryzae* for explants collected from all the sites except site 1 of Khowai District (*Fusarium equiseti*) in fruiting season (Table 4). The highest relative frequency was observed in case of *F. equiseti* (Site 1 and 2 of Sepahijala District and site 1 of Khowai District) and for *Diaporthe rosae* (Site 1 and 2 of West Tripura and Dhalai District and site 2 of Khowai District) in non-fruiting season (Table 4). The relative frequencies of isolated fungi of *A. heterophyllus* in fruiting (sites: F = 12.22, $p < 0.001$) and non-fruiting season (sites: F = 10.49, $p < 0.001$) significantly differed among the eight sampling sites, whereas relative frequencies of specific endophytic fungal strain showed no significant differences among different sampling sites.

In *C. reticulata* highest Simpson, Shannon and Evenness values were recorded in foliar explants of South district sampled in non-fruiting season. But Dominance value was maximum in explants of Sepahijala district in both the seasons (Table 5).

In *A. heterophyllus* highest Simpson, Shannon and Evenness values were recorded in foliar explants of site 1 of Dhalai District sampled in non-fruiting season. But dominance value was maximum in explants of Dhalai district sampled in fruiting season (Table 6 and 7).

It was observed in an earlier study that for *C. reticulata*, endophytic colonization among various sampling sites differs. Fungal colonization rate

Table 5. Diversity indices of foliar endophytic fungi isolated from *C. reticulata* in fruiting and non-fruiting seasons

Indices	Fruiting Season			Non-fruiting Season		
	GC1	SEC1	SC1	GC1	SEC1	SC1
Taxa_S	7	7	7	7	7	7
Dominance_D	0.1581	0.1731	0.1626	0.1643	0.1731	0.1556
Simpson_1-D	0.8419	0.8269	0.8374	0.8357	0.8269	0.8444
Shannon_H	1.894	1.846	1.884	1.873	1.837	1.903
Evenness_e^H/S	0.9494	0.905	0.9399	0.93	0.8968	0.9578

Note: GC1: Gomati *Citrus* 1, SC1: South *Citrus* 1, SEC1: Sepahijala *Citrus* 1

Table 6. Diversity indices of foliar endophytic fungi isolated from *A. heterophyllus* in fruiting season from different sites

Indices	WA1	WA2	SEA1	SEA2	KA1	KA2	DA1	DA2
Taxa_S	4	5	5	5	5	5	4	5
Dominance_D	0.3389	0.2517	0.2628	0.2934	0.3152	0.3062	0.345	0.2817
Simpson_1-D	0.6611	0.7483	0.7372	0.7066	0.6848	0.6938	0.655	0.7183
Shannon_H	1.227	1.489	1.461	1.403	1.342	1.37	1.208	1.423
Evenness_e^H/S	0.8524	0.8867	0.8623	0.8133	0.7652	0.7871	0.8367	0.8297

Note: WA1: West *Artocarpus* 1, WA2: West *Artocarpus* 2, SEA1: Sepahijala *Artocarpus* 1, SEA2: Sepahijala *Artocarpus* 2, KA1: Khowai *Artocarpus* 1, KA2: Khowai *Artocarpus* 2, DA1: Dhalai *Artocarpus* 1, DA2: Dhalai *Artocarpus* 2

Table 7. Diversity indices of foliar endophytic fungi isolated from *A. heterophyllus* in non-fruiting season from different sites.

Indices	WA1	WA2	SEA1	SEA2	KA1	KA2	DA1	DA2
Taxa_S	5	5	5	5	5	5	5	6
Dominance_D	0.2553	0.2892	0.3056	0.2864	0.3347	0.2521	0.2438	0.248
Simpson_1-D	0.7447	0.7108	0.6944	0.7136	0.6653	0.7479	0.7562	0.752
Shannon_H	1.481	1.403	1.356	1.415	1.334	1.484	1.504	1.567
Evenness_e^H/S	0.8795	0.8134	0.7764	0.823	0.7592	0.8817	0.9001	0.7986

Note: WA1: West *Artocarpus* 1, WA2: West *Artocarpus* 2, SEA1: Sepahijala *Artocarpus* 1, SEA2: Sepahijala *Artocarpus* 2, KA1: Khowai *Artocarpus* 1, KA2: Khowai *Artocarpus* 2, DA1: Dhalai *Artocarpus* 1, DA2: Dhalai *Artocarpus* 2

and colonization frequency (%) value were also influenced by the sampling season. *Penicillium citrinum* was isolated as a foliar endophyte from *C. reticulata*. Diversity indices i.e. α-diversity also differed between sampling sites and seasons. As suggested, the ecological and environmental conditions might affect the colonization of host tissues by endophytes [31]. In a previous report, *C. gloeosporioides* and *Epiccocum nigrum* were isolated as endophytes from *C. sinensis*. However, the season of sampling influenced the abundance of fungal species and diversity of fungal communities. Most of the isolated fungal strains belonged to Ascomycota [32]. These findings correlated with the present experimental results. The previous report documented that *E. nigrum* was isolated from *C. sinensis* and *P· citrinum* from *C. reticulata*, *Nodulisporium* sp., *Pestalotiopsis* sp., and *Xylaria* sp. as endophyte from *C. limon* [33] which corresponded with present experimental findings. Besides these, The similarity in endophytic assemblages isolated from explants of different sampling sites were observed in the present investigation. This might be supported by previous report which also identified such similarity in distribution of fungal endophytes in Ananus comosus, another fruit yielding plant from Tripura [34].

Species of *Aspergillus*, *Colletotrichum*, *Diaporthe*, were isolated as foliar endophytes of *A. heterophyllus* [35]. *Diaporthe phaseolorum* isolated as endophyte from healthy leaf tissues of *A. heterophyllus* [36]. In the present investigation, other species of the *Diaporthe* (*D. rosae*), *Aspergillus* (*A. japonicus* and *A. fumigatus*) and *Colletotrichum artocarpi* were isolated as foliar endophytes. *Alternaria alternata*, *C. artocarpi*, and *Fusarium oxysporum* were isolated previously

from the leaf of *A. heterophyllus*, which correlated with the present findings [37]. In addition, it was reported that *Pestalotiopsis microspora* was isolated from the stem of *A. heterophyllus* as an endophytic fungus [38]. In this study, *P. versicolor* was recovered as foliar endophytes from the *A. heterophyllus*.

It was suggested that the sampling site might be an important determinant of the fungal community associated with the host plant. Altered microclimates in different locations influence the host plant species as well as endophytic fungal composition [35]. In current investigation composition of foliar fungal endophytes in two host plants varied. Moreover, in different sampling sites, fungal compositions also differed in the case of *A. heterophyllus*.

C. gloeosporioides and *Phomopsis* are known pathogen of *C. reticulata* [39] but isolated as foliar endophytes in this study from *C. reticulata*. Further, it was stated that *C. gloeosporioides* is the agent of citrus anthracnose, but also known for their endophytic aptitude [33]. This can be explained by the statement of the previous report that host and fungus genotypes with environmental conditions influence the direction of interactions between plant and fungal strains [40]. The genotype and developmental stage of the host plant primarily govern the endophytic community assemblage [41]. The statement agrees with the present results where the type of host plants along with fruiting and non-fruiting stage influenced the community composition of foliar endophytes. According to Paul *et al.* [42], the Simpson and Shannon diversity indices differed among seedling stage, flowering and fruiting stages of *Capsicum annuum* which corresponded with present findings where diversity indices varied between fruiting and non-fruiting stages of host plants. According to Sánchez-Márquez *et al.* [43] increase in the number of sampled plants or locations would have a possibility to reveal new fungal endophytic taxa. Thus, increase in sampling sites might also increase the diversity of fungal strains in two selected host plants.

Conclusion

Extensive sampling of host plant from other sampling sites may be required for isolation of some other endophytic fungal strains with novel properties. The observation and results of the present study may help to assess the antagonistic properties of isolated endophytic fungi to develop biocontrol agents against plant pathogens. Endophytic fungi isolated from the host plants may be assessed for enhancing the growth of crop plants and also for increasing shelf life of fruits of the host plants.

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