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Article

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Mycoendophytic diversity and their antimicrobial potential from two epiphytic orchids of the Western Ghats forests of India

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Abstract

The epiphytic plants belong to a group that harmlessly grows on other plants by utilizing the nutrition from the host plants with their unique adaptation features along with symbiotic associations with fungi or bacteria. The various biological activities exhibited by the mycoendophytes inhabiting medicinally-important epiphytic orchids serve as the primary source of novel drug leads, industrially-essential enzymes, and plant growth-promoting metabolites. In the present study, a total of 956 culturable mycoendophytes out of 1600 segments belonging to 17 genera were isolated from different tissue parts of *Trias stocksii* and *Dendrobium herbaceum*. The Xylariaceae taxa were the predominant mycoendophytes present in both plants, followed by *Pestalotiopsis* sp., *Colletotrichum* sp., and *Fusarium* sp. An estimation of the Shannon–Wiener and Simpson diversity indices showed that the bulbs of *T. stocksii* have the highest species diversity index and the stems of *D. herbaceum* the lowest. The highest species richness was observed in the leaves of *T. stocksii* and the lowest in the leaves of *D. herbaceum*. Overall, *T. stocksii* harbored more mycoendophytes along with the highest diversity indices compared to *D. herbaceum*. The antimicrobial evaluation revealed that *Xylaria* sp. has a higher potential of producing anti-infectives and opens a new arena for industrial exploration.

Key words – Antimicrobial activity – *Dendrobium herbaceum* – Diversity indices – Mycoendophytes – *Trias stocksii* – *Xylaria* sp.

Introduction

Unique adaptation characteristics such as symbiotic associations with fungi or bacteria and the presence of specific aerial root systems are found in the majority of Orchidaceae members that primarily exist as epiphytes (Hossain et al. 2013, Parthibhan et al. 2017). Many species of epiphytic orchids have been exploited for their ethnobotanical importance in traditional medicine – to cure gastritis infections, cancer, aging, and syphilis – making them a unique resource for herbal drugs (Li et al. 2009, Chen et al. 2013). It has triggered the search by natural product researchers, for bioactive potentials to combat various infections and diseases.

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The mycoendophytes represent endophytic fungal communities associated with plants. From seedlings to well-grown orchids, the interaction of mycorrhizal and non-mycorrhizal mycoendophytes in various biological events has been previously reported (Rasmussen & Whigham 2002, Dearnaley et al. 2012, Freudenstein & Chase 2015, Rasmussen et al. 2015, Herrera et al. 2017). The symbiotic association between orchids and mycoendophytes has been considered as mutualism, where the mycoendophytes benefit the relationship by providing greater access to water and mineral ions to the plant, some are capable of producing plant growth-accelerating and protection molecules (Mapperson et al. 2014, Ye et al. 2014, Khamchatra et al. 2016). Many Orchidaceae members are associated with single mycoendophytes, while others harbor several numbers; also, there are changes in the association according to environmental influences (Da Silva et al. 2015, Rasmussen et al. 2015). For decades, the importance of mycoendophytes has been exploited in major areas including drug development, plant growth-promotion as well as protection, and production of industrially-essential enzymes (Aly et al. 2010, Demain 2014, Harvey et al. 2015, Macías-Rubalcava & Sánchez-Fernández 2017, Shubha & Srinivas 2017).

The primary step in the search for bioactive potentials from mycoendophytic communities is to identify and assess the diversity in different parts of the host. In this study, for the first time, we discuss the morphological identification, assessment of the mycoendophytic diversity, and their antimicrobial efficacy associated with two epiphytic orchids of the Western Ghats of Karnataka, India, namely, *Dendrobium herbaceum* Lindl. and *Trias stocksii* Benth. ex Hook.f. To the best of our belief, this is the first report on the evaluation of the diversity and antimicrobial profiling of mycoendophytes associated with *Trias stocksii*, which is an endemic epiphytic orchid distributed in the Western Ghats region of India.

Materials & Methods

Materials

The culture media used for the isolation of mycoendophytes and the maintenance of pure culture along with the standard antibiotics were procured from HiMedia (Mumbai, India). The sodium hypochlorite solution (with 4 % available chlorine) was acquired from Fisher Scientific (Mumbai, India).

Study site and sampling

The healthy samples of the epiphytic orchids *Dendrobium herbaceum* and *Trias stocksii* were gathered from Kigga village, Sringeri (Western Ghats region) at 13°24'59.2" N 75°10'49.9" E and 13°24’50.8” N 75°11’01.7” E respectively. They were collected in separate sterile polythene bags and processed within 12 hours.

Isolation of mycoendophytes

The collected plant samples were washed thoroughly under running tap water and air-dried. The healthy parts were cut into 0.5 cm^2 bits before subjecting them to a surface sterilization process in which the bits were treated with 70 % alcohol for 1 min followed by blot drying, 4% sodium hypochlorite solution for 4–5 min followed by blot drying and three sequential washes with sterile distilled water followed by blot drying. The surface-sterilized tissue bits were placed equidistantly on water agar plates (10 bits per plate) previously supplemented with antibiotic chloramphenicol (200 mg L^{-1}). The inoculated plates were incubated at 25 ± 2°C for 5–7 days under 12 hr respectively of light and dark cycles. The pure cultures of young mycelium emerging from the tissue bits were transferred on to PDA (potato dextrose agar) plates amended with chloramphenicol (200 mg L^{-1}) using a sterile needle, and the plates were incubated at 25 ± 2°C for 5–7 days under 12 hr respectively of light and dark cycles (Kumara et al. 2014, Rakshith et al. 2016).

Morphology-based identification

The identification of mycoendophytes was carried out using the microscopic (Olympus

CX41, Japan) and cultural characteristics based on previously-published illustrations and standard mycological monographs. The isolates were grouped according to their genus and cataloged using alphanumerical characters; the mycelia, which failed to sporulate were grouped under Mycelia sterilia (Sutton 1980, Barnett & Hunter 1998, Mathur & Kongsdal 2003, Leslie et al. 2006).

Data analysis

The quantification of the culturable mycoendophytic inhabitants of the epiphytic orchids was done as follows: (i) the colonization frequency (CF) was evaluated as the number of tissue segments colonized by a specific mycoendophyte divided by the total number of tissue segments observed and was expressed as a percentage, (ii) the isolation rate (IR) was calculated as the total number of mycoendophytes isolated divided by the total number of tissue segments placed (Jinu & Jayabaskaran 2015).

Mycoendophytic diversity

The dominance of mycoendophytes was determined using Camargo's Index (1/S), where S represents the total species richness in the community. The diversity indices such as Shannon (H) and Simpson (1-D) indices were estimated for the mycoendophytic inhabitants using $H = -\Sigma pi \ln (pi)$ and $D = \Sigma (pi)^2$ respectively, where pi is the proportion of mycoendophytes that i contributes to the total. The evenness (E) was expressed as $E = H / \ln(S)$ (Suryanarayanan & Kumaresan 2000, Suryanarayanan et al. 2009, Dhayanithy et al. 2019).

Ecological associations

The ecological interrelationships between the mycoendophytes and the different tissue types of both epiphytic orchids were analyzed by principal component analysis (PCA) using the Origin software (Version 2018) (Rivera-Orduña et al. 2011).

Antimicrobial profiling by agar plug assay

Antimicrobial efficacy of isolated mycoendophytes was tested by agar plug diffusion assay against a Gram-positive bacteria *Staphylococcus aureus*, a Gram-negative bacteria *Escherichia coli* and a dermatophyte *Candida albicans* with minor modifications. Agar plugs of 21 days old mycoendophytic isolates were placed on the respective medium previously seeded with the test microbial pathogens (adjusted to 0.5 McFarland standard). Inoculated plates were then incubated at 8°C for 30 min and then at 37°C for 24 hr for bacteria and 25 ± 2°C for 48-72 hr for dermatophyte respectively. After incubation, based on the presence or absence of the inhibition zone around the agar plugs, antimicrobial profiling was assessed (de Siqueira et al. 2011).

Results and discussion

Isolation and identification of mycoendophytes

The mutualistic interactions between epiphytic orchids and mycoendophytes have been reported extensively in the past few decades around the globe. Along with multiple ecological roles, the mycoendophytes of epiphytic orchids are well-recognized for their biological potentials. One of the biodiversity hotspots in India – the Western Ghats – is considered as the home for various flora with bioactive potentials that need to be explored (Bose et al. 2019). The mycoendophytes inhabiting epiphytic orchids were found to be least exploited in the Western Ghats, and an attempt was made in this study to isolate and identify the diverse mycoendophytes inhabiting the epiphytic orchids *D. herbaceum* and an endemic *T. stocksii* (Fig. 1). This endemic *T. stocksii* was earlier termed as *Bulbophyllum stocksii* (Sinu et al. 2011, Vermeulen et al. 2014, Mathew & George 2015). A total of 956 culturable mycoendophytes belonging to 17 different genera were isolated from the 1600 tissue bits of both the selected epiphytic orchids. The most abundant among the isolated mycoendophytes was *Xylaria* sp., followed by *Pestalotiopsis* sp. and *Colletotrichum* sp.

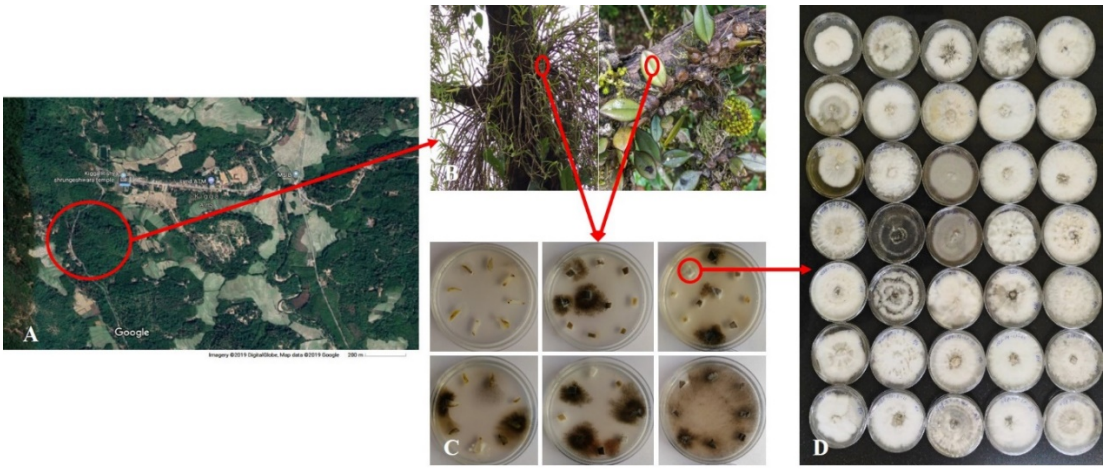

Fig. 1 – Overall view of Mycoendophytes isolation. A Location of the sampling site. B Habitat of the selected epiphytic orchids *Dendrobium herbaceum* (Left) and *Trias stocksii* (Right). C Growth of mycoendophytes on water agar plates. D Pure cultures of mycoendophytes on PDA plates.

The mycoendophytes isolated from ten different medicinal *Dendrobium* sp. by Chen et al. (2011) recorded a total of 401 isolates in which *Fusarium* sp. was the highest isolated mycoendophyte followed by *Acremonium* sp., *Alternaria* sp., *Colletotrichum* sp. and *Verticillium* sp. In the present study, the endemic epiphytic orchid *Trias stocksii* harbored the majority of mycoendophytes (524 isolates) with respect to *Dendrobium herbaceum* (432 isolates). Based on the morphological and microscopic observations, the mycoendophytes were grouped into 17 genera, and the ones that failed to produce mitosporic features were cataloged as Mycelia sterilia (Table 1, Fig. 2). The mycoendophytes that belongs to the class Sordariomycetes (75.10 %) were extensively associated with Orchidaceae members. Among Sordariomycetes, *Xylaria* sp. and *Pestalotiopsis* sp. were the highest isolates recovered from both the orchids. The highest number of individual mycoendophytic species were Sordariomycetes (75.10 % of the total isolates) – thirteen and twelve different species from *D. herbaceum* and *T. stocksii* respectively, followed by Dothideomycetes (14.85 %), Eurotiomycetes (8.15 %) and Mycelia sterilia (1.88 %) (Fig. 3).

The association of Xylariaceae members as endophytes has been reported extensively in epiphytic orchids of the genus *Dendrobium* (Chen et al. 2011, 2013). The colonization frequency was observed to be highest in *T. stocksii* (58%) compared to *D. herbaceum* (45.5%). Among the individual mycoendophytes recovered from both the epiphytic orchids, *Xylaria* sp. had the highest number of individuals with a CF of 30.5%, followed by *Pestalotiopsis* sp. (4%), *Colletotrichum* sp. (3.25%), *Fusarium* sp. (2.87%) and *Alternaria* sp. (2.50%), with the least CF observed in *Botryodiplodia* sp. (0.37%) (Table 2).

Mycoendophytic diversity

The foliar mycoendophytic diversity is selectively higher when compared to other tissues in the flora found in tropical forests (Arnold 2007, Arnold & Lutzoni 2007). The association and diversity indices of mycoendophytes have been studied in a wide range of ethnomedicinal plants from the Western Ghats (Raviraja 2005, Naik et al. 2008, Nalini et al. 2014). An evaluation of the root mycoendophytes associated with four epiphytic orchids, reported by de los Angeles Beltrán-Nambo et al. (2018) showed less values when compared to the foliar endophytes. A previous study reported that foliar mycoendophytes are highest in number when compared to roots of the epiphytic orchids *Bulbophyllum neilgherrense* and *Vanda testacea* (Sudheep & Sridhar 2012). The non-mycorrhizal endophytes from the leaves of epiphytic orchids showed a higher colonization frequency when compared to the roots of *Bulbophyllum neilgherrense* and *Pholidota pallida* (Sawmya et al. 2013). In the present study, the focus was on the evaluation of foliar mycoendophytic diversity rather than isolates from the root inhabitants.

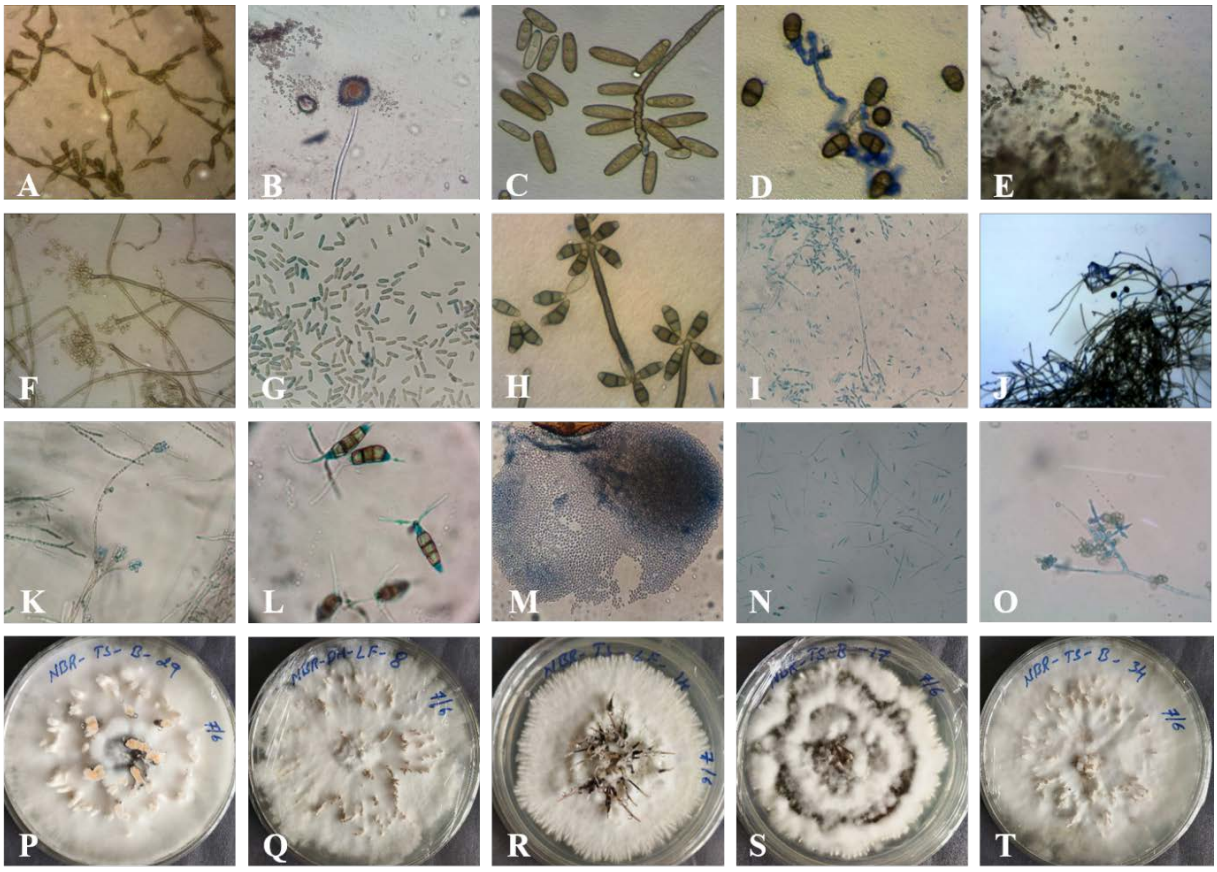

Fig. 2 – Microscopic images of A *Alternaria* sp. B *Aspergillus* sp. C *Bipolaris* sp. D *Botryodiplodia* sp. E *Chaetomium* sp. F *Cladosporium* sp. G *Colletotrichum* sp. H *Curvularia* sp. I *Fusarium* sp. J *Nigrospora* sp. K *Penicillium* sp. L *Pestalotiopsis* sp. M *Phoma* sp. N *Phomopsis* sp. O *Trichoderma* sp. P-T cultural characteristics of different *Xylaria* sp. producing ecto-stromata.

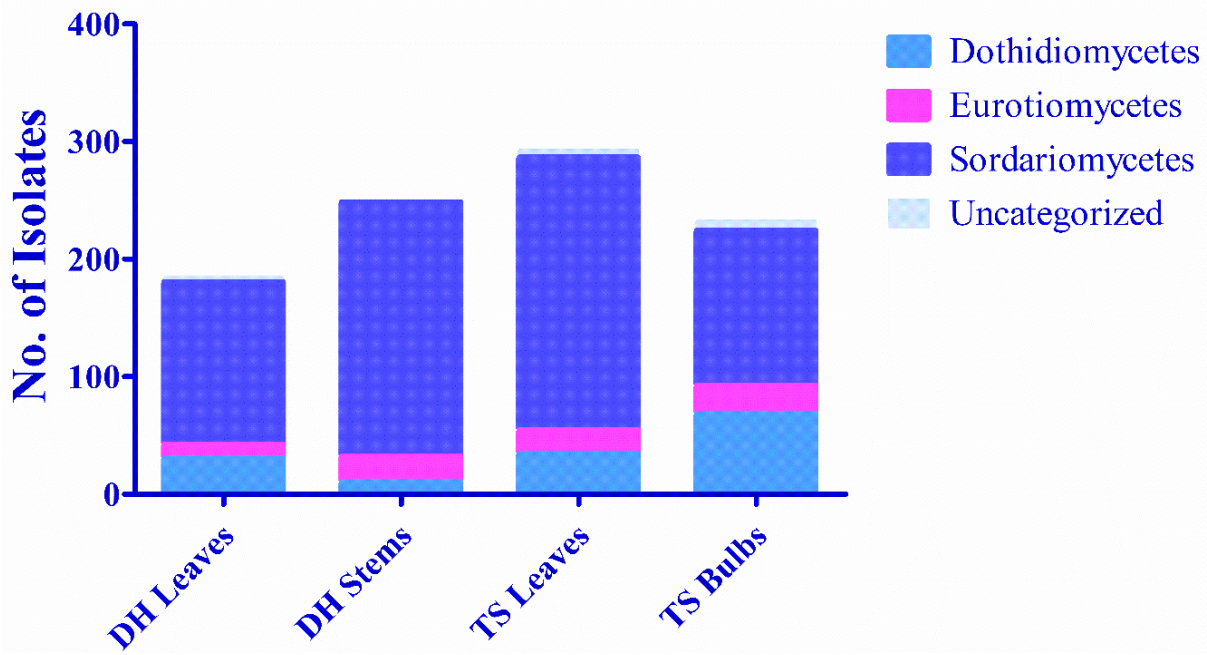

Fig. 3 – Classwise distribution of isolated mycoendophytic communities from *D. herbaceum* and *T. stocksii*.

Table 1 Distribution and Colonisation frequency (CF %) of mycoendophytic communities from *D. herbaceum* and *T. stocksii*.

Sl. No.	Class	Family	Fungi	*Dendrobium herbaceum*		*Trias stocksii*		Total	CF (%)
				Leaves	Stems	Leaves	Bulbs		
1.	**Dothidiomycetes**	Davidiellaceae	*Cladosporium* sp.	00	04	04	08	016	**01.00**
2.		Botryosphaeriaceae	*Botryodiplodia* sp.	00	00	06	00	006	**00.37**
3.		Incertae sedis	*Phoma* sp.	06	00	10	20	036	**02.25**
4.			*Alternaria* sp.	12	06	10	12	040	**02.50**
5.		Pleosporaceae	*Bipolaris* sp.	00	00	00	12	012	**00.75**
6.			*Curvularia sp.*	12	00	04	16	032	**02.00**
7.	**Eurotiomycetes**	Trichocomaceae	*Aspergillus* sp.	08	10	08	08	034	**02.12**
8.			*Nigrospora* sp.	00	04	06	00	010	**00.62**
9.			*Penicillium* sp.	04	08	06	16	034	**02.12**
10.	**Sordariomycetes**	Amphisphaeriaceae	*Pestalotiopsis* sp.	14	12	18	20	064	**04.00**
11.		Chaetosphaeriaceae	*Chaetomium* sp.	10	08	10	08	036	**02.25**
12.		Diaporthaceae	*Phomopsis* sp.	00	08	00	08	016	**01.00**
13.		Glomerellaceae	*Colletotrichum* sp.	14	10	12	16	052	**03.25**
14.		Hypocreaceae	*Trichoderma* sp.	00	10	06	00	016	**01.00**
15.		Nectriaceae	*Fusarium* sp.	10	06	14	16	046	**02.87**
16.		Xylariaceae	*Xylaria* sp.	90	162	172	64	488	**30.50**
17.	**Uncategorized**	Mycelia sterilia	Morpho sp.	04	000	006	08	018	**01.12**
	Total No. of isolates			**184**	**248**	**292**	**232**	**956**	**59.75**

Table 2 Data analysis of isolated mycoendophytic communities from *D. herbaceum* and *T. stocksii*.

Plants	Tissue sample	No. of Segments Plated	No. of segments yielding endophytic fungi	No. of isolates	Isolation rate	Colonization Frequency (%)
Dendrobium herbaceum	**Leaves**	400	162	184	0.46	40.5
	Stems	400	202	248	0.62	50.5
Trias stocksii	**Leaves**	400	260	292	0.73	65
	Bulbs	400	204	232	0.58	51
Total		**1600**	**808**	**956**	**0.59**	**51.75**

The mycoendophytic diversity analysis was done with the Shannon–Wiener (H) and Simpson (1-D) indices, they were observed to be highest (2.40 and 1.45 respectively) in the bulbs of *T. stocksii* and lowest (0.87 and 0.56 respectively) in the stem tissues of *D. herbaceum*, these values are less than those in *B. neilgherrense* and *V. testacea*. The species richness and total abundance were observed to be highest (292 and 184 respectively) in the leaves of *T. stocksii* and lowest (15 and 11 respectively) in the leaves of *D. herbaceum*, in comparison, the mycoendophytes from the leaves of *V. testacea* was found to be 15, and the least was observed in the bulb tissues of *B. neilgherrense*. The highest value of evenness (0.91) was observed in the bulb tissues of *T. stocksii* as compared to the other tissues of *T. stocksii* and *D. herbaceum* along with the mycoendophytes of *B. neilgherrense* and *V. testacea* (Table 3) (Sudheep & Sridhar 2012).

Several reports of mycoendophytic diversity with seasonal variations from different medicinal plants of the Western Ghats suggest the dominance of *Fusarium* sp., *Alternaria* sp., *Acremonium* sp., *Pestalotiopsis* sp., *Curvularia* sp. and *Colletotrichum* sp. (Raviraja 2005, Naik et al. 2008, Sudheep & Sridhar 2012, Nampoothiri et al. 2013, Nalini et al. 2014). The present study is the first report on the dominance of Xylariaceae among the epiphytic orchids of the Western Ghats. The aforementioned results correlate with the results of Chen et al. (2011, 2013) in which *Fusarium* sp., *Alternaria* sp., *Verticillium* sp., and *Xylaria* sp. were the dominant fungal genera from 10 *Dendrobium* medicinal plants.

Table 3 Diversity indices calculation of mycoendophytic communities from *D. herbaceum* and *T. stocksii*.

Plants	Tissue sample	Total Abundance	Species Richness (*S*)	Camargo's Index (1/*S*)	Diversity		Evenness (*E*)
					Shannon-Wiener (*H*)	Simpson (1-*D*)	
Dendrobium herbaceum	Leaves	184	11	0.09	1.82	0.73	0.76
	Stems	248	12	0.08	1.45	0.56	0.58
Trias stocksii	Leaves	292	15	0.07	1.72	0.63	0.63
	Bulbs	232	14	0.07	2.40	0.87	0.91

Ecological associations

The mycoendophytic associations of different parts of *Taxus globosa* showed that 78% of the total variations were with two components of the PCA, among the mycoendophytes isolated, Xylariaceae members were found in all the tissues of *T. globosa* (Rivera-Orduña et al. 2011). An analysis of the two principal components obtained from PCA showed 96.22% of the total variance. Thus, the overall PCA revealed that the majority of mycoendophytes were somewhat evenly distributed among the different tissue types of both the epiphytic orchids. Some mycoendophytes showed tissue specificity in both orchids. For example, *Botryodiplodia* sp. was found to be explicitly associated with the leaves of *T. stocksii* and *Bipolaris* sp. was found to be associated with the bulbs of *T. stocksii*. While *Phomopsis* sp. was recovered from the stems of *D. herbaceum* and the bulbs of *T. stocksii*, it failed to be isolated from the foliar parts of both the epiphytic orchids. In comparison, *Xylaria* sp., *Pestalotiopsis* sp., *Colletotrichum* sp., *Fusarium* sp., and *Alternaria* sp. were mainly found in all the tissue parts of *D. herbaceum* and *T. stocksii* (Figs 4–5).

Antimicrobial profiling

Host plant protection by producing a plethora of bioactive antimicrobial secondary metabolites is one of the beneficial aspects of the symbiotic relationship with the mycoendophytes and which can be further exploited for industrial application (Macías-Rubalcava & Sánchez-Fernández 2017). Antimicrobial profiling of the isolated mycoendophytes provides an overview of the selection of the bioactive isolates for further industrial applications. Initial screening of antimicrobial activity for the selection of bioactive isolates plays a crucial role in the identification of bioactive mycoendophytes. Overall, 956 mycoendophytic isolates were cultured on the PDA

plates without any antibiotic supplements for the antimicrobial profiling by agar plug assay. Among 956 mycoendophytic isolates, 34 isolates (13 isolates of *D. herbaceum* and 21 isolates of *T. stocksii*) belongs to 17 different fungal genera inhibited at least one or more tested microbial pathogens (*Staph. aureus*, *E. coli*, and *C. albicans*) as represented in Table 4, Fig. 6. Selected bioactive mycoendophytic cultures can be further processed for the isolation of potent broad-spectrum anti-infective drug leads.

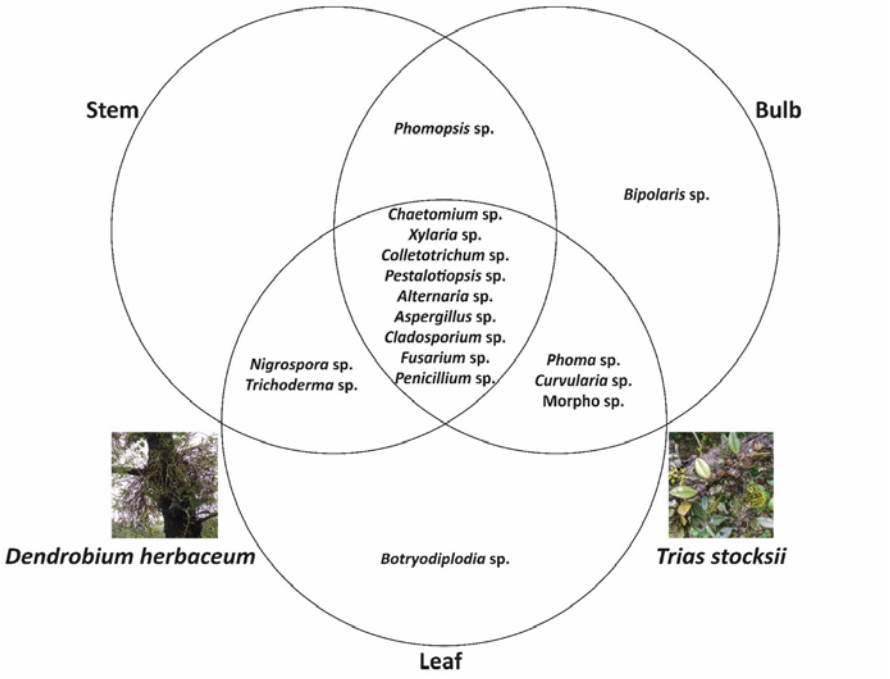

Fig. 4 – Common endophytic fungal genera comparison between different tissues of *D. herbaceum* and *T. stocksii*.

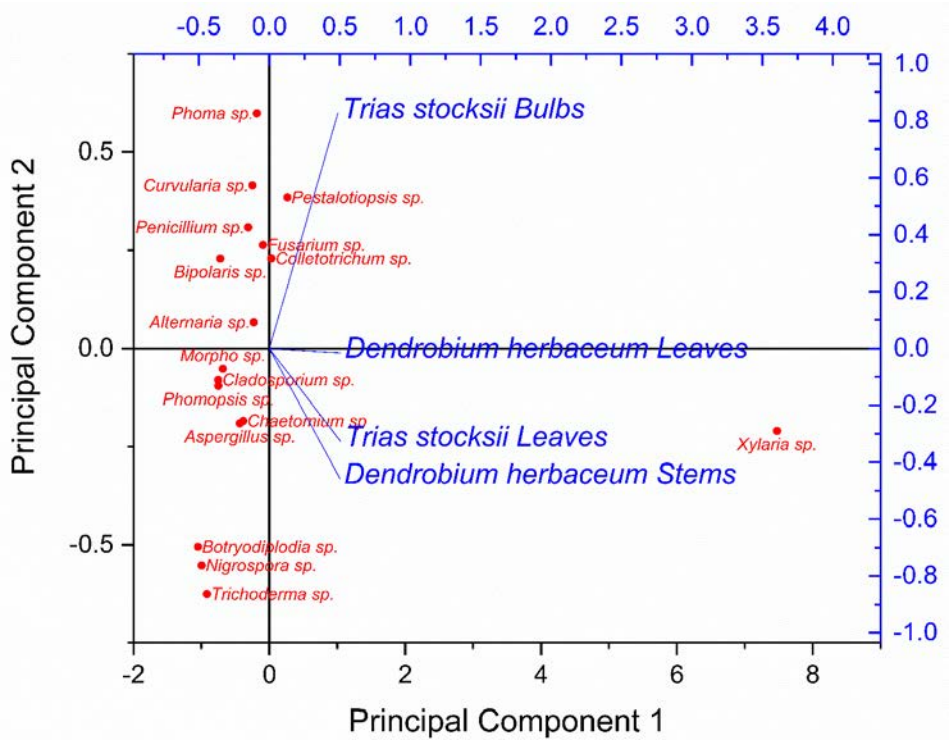

Fig. 5 – Principal Component Analysis (PCA) of mycoendophytes isolated from different tissues of *D. herbaceum* and *T. stocksii.*

Table 4 Antimicrobial Profiling of mycoendophytes using agar plug diffusion assay.

Fungal endophyte	Isolate No.	Inhibited microorganisms	Antimicrobial activity
Alternaria sp.	NBRDHLF- 54	*Staph. aureus*	+
Aspergillus sp.	NBRDHST- 74	*Staph. aureus*	+
Bipolaris sp.	NBRTSB- 14	*E. coli*	+
Botryodiplodia sp.	NBRTSLF- 07	*Staph. aureus*	+
Chaetomium sp.	NBRDHLF- 36	*Staph. aureus*	+
Cladosporium sp.	NBRTSB- 68	*Staph. aureus*	+
Colletotrichum sp.	NBRDHLF- 67	*Staph. aureus* and *E. coli*	++
Colletotrichum sp.	NBRTSB- 37	*E. coli*	+
Curvularia sp.	NBRDHLF- 12	*Staph. aureus*	+
Curvularia sp.	NBRTSB- 52	*Staph. aureus*	+
Fusarium sp.	NBRDHST- 58	*Staph. aureus* and *E. coli*	++
Fusarium sp.	NBRTSLF- 04	*Staph. aureus*	++
Fusarium sp.	NBRTSB- 28	*Staph. aureus* and *E. coli*	+
Morpho sp.	NBRTSLF- 15	*Staph. aureus*	+
Nigrospora sp.	NBRTSLF- 26	*Staph. aureus*	+
Penicillium sp.	NBRDHST- 19	*Staph. aureus*	+
Penicillium sp.	NBRTSB- 24	*Staph. aureus*	+
Pestalotiopsis sp.	NBRDHLF- 05	*Staph. aureus*	+
Pestalotiopsis sp.	NBRDHST- 37	*Staph. aureus* and *E. coli*	++
Pestalotiopsis sp.	NBRTSLF- 43	*Staph. aureus*	+
Phoma sp.	NBRTSLF- 64	*Staph. aureus*	+
Phomopsis sp.	NBRDHST- 06	*Staph. aureus* and *E. coli*	+
Trichoderma sp.	NBRDHST- 11	*Staph. aureus* and *E. coli*	++
Trichoderma sp.	NBRTSLF- 57	*Staph. aureus*	+
Xylaria sp.	NBRDHST- 45	*Staph. aureus* and *E. coli*	++
Xylaria sp.	NBRDHST- 26	*Staph. aureus* and *E. coli*	++
Xylaria sp.	NBRTSB- 20	*Staph. aureus, E. coli* and *C. albicans*	+++
Xylaria sp.	NBRTSB- 43	*Staph. aureus, E. coli* and *C. albicans*	+++
Xylaria sp.	NBRTSB- 17	*Staph. aureus, E. coli* and *C. albicans*	+++
Xylaria sp.	NBRTSB- 23	*Staph. aureus, E. coli* and *C. albicans*	+++
Xylaria sp.	NBRTSB- 35	*Staph. aureus, E. coli* and *C. albicans*	+++
Xylaria sp.	NBRTSB- 54	*Staph. aureus, E. coli* and *C. albicans*	+++
Xylaria sp.	NBRTSLF- 58	*Staph. aureus, E. coli* and *C. albicans*	+++
Xylaria sp.	NBRTSLF- 18	*Staph. aureus* and *E. coli*	++

+: The zone of inhibition in diameter is less (<) than 10 mm
++: The zone of inhibition in diameter is between 11 to 15 mm
+++: The zone of inhibition in diameter more (>) than 15 mm

Conclusion

The findings from the overall diversity analysis suggest that the mycoendophytes inhabiting both the epiphytic orchids of the Western Ghats were evenly distributed among the plants with little variation. The data reported supports the co-existence of mycoendophytes, with symbiotic associations, among both the epiphytic orchids. An analysis of the various diversity indices revealed that the fungi belonging to the genus *Xylaria* were dominant in both the epiphytic orchids. Antimicrobial profiling of the mycoendophytes revealed that bioactive isolates had the broad-spectrum antimicrobial activity. This is the first study reporting the diversity and antimicrobial efficacy of mycoendophytes inhabiting the epiphytic orchid *D. herbaceum* and an endemic *T. stocksii* from the Western Ghats of Southern India.

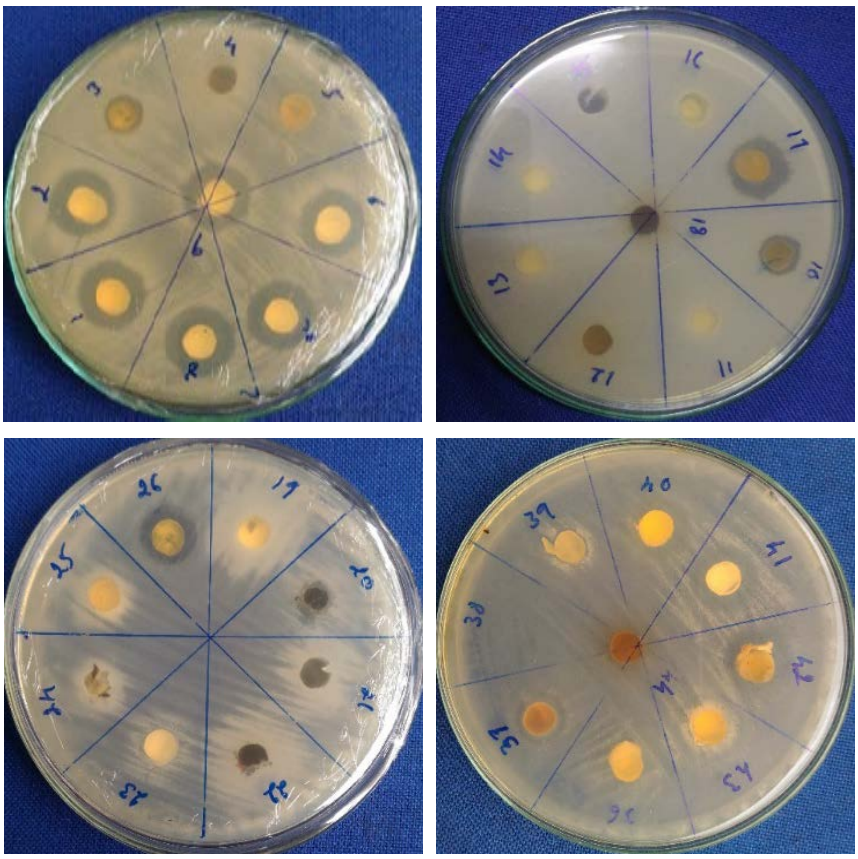

Fig. 6 – Zone of inhibition around the bioactive mycoendophytic agar plugs.

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Conflict of interest

The authors have declared no potential conflict of interest.

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