Fungal Biology 123 (2019) 373–383

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Community structure, spatial distribution, diversity and functional characterization of culturable endophytic fungi associated with *Glycyrrhiza glabra* L.

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ARTICLE INFO

Article history:
Received 23 July 2018
Received in revised form
22 December 2018
Accepted 13 February 2019
Available online 18 February 2019

Corresponding Editor: Angel Medina

Keywords:
Co-culture
Endophyte
Himalayas
Licorice
Plant-microbe interactions

ABSTRACT

A total of 266 endophytic fungal isolates were recovered from 1019 tissue segments of *Glycyrrhiza glabra* collected from four different locations in the North-Western Himalayas. The endophytes grouped into 21 genera and 38 different taxa. The host had strong affinity for the genus *Phoma*, followed by *Fusarium*. The species richness was highest at the sub-tropical location, followed by the sub-temperate location and the temperate locations, respectively. The tissue specificity of endophytes was also evident. Some endophytes showed potential antimicrobial activity against phyto-pathogens indicating that they may be helpful to the host in evading pathogens. All the endophytic taxa produced the plant growth promoting hormone, indole acetic acid (IAA), though in varying concentrations. None of these endophytes caused any symptoms of disease in co-cultivation with the tissue cultured plants. Further, all the endophytes had a positive influence on the phenolic and flavonoid content of the host. Three endophytes, *Stagonosporopsis cucurbitacearum*, *Bionectria* sp. and *Aspergillus terreus* also increased the host root (rhizome) and shoot growth visibly. Such endophytes are potential candidates for developing endophyte-based technologies for sustainable cultivation and enhanced productivity of *G. glabra.* This is the first report of community structure and biological properties of fungal endophytes associated with *G. glabra.*

1. Introduction

In natural ecosystems, all plants are in symbiosis with endophytes which significantly influence the plant community structure and diversity (Wani et al., 2015). The endophytes while harbored in different plant communities produce exclusive compounds of therapeutic value or they might as well produce the compounds which mimic some secondary metabolites produced by the host plant (Kusari et al., 2014). There are also reports where endophytes are shown to induce or at least enhance production of secondary metabolites by the host plant (Kusari et al., 2014). Thus, the plant-endophyte interface provides an ecological marketplace for harnessing the potential of endophytes to produce compounds of therapeutic potential or exert their positive influence on plants to enhance the production of specialized metabolites of plant origin.

Glycyrrhiza glabra L. (liquorice), a member of the Leguminosae family, is an important medicinal plant used traditionally over centuries to treat human illness (Hosseinzadeh and Nassiri-Asl, 2015). *Glycyrrhiza* is known for promising antitumor, antimicrobial, antiviral, anti-inflammatory, anti-diabetic, immunoregulatory, hepatoprotective and neuroprotective activities (Park et al., 2004; Yang et al., 2017). The pharmaceutical importance of *Glycyrrhiza* lies in its capacity to produce a variety of secondary metabolites comprising of terpenes, flavonoids, isoflavonoids, chalcones, coumarins and polysaccharides (Seki et al., 2011). *Glycyrrhiza* also produces an array of volatile components out of which about 35 % are terpenoids with octanoic acid, paeonol, octadecane, benzaldehyde, α-terpineol and 4-terpineol as its main constituents (Miyazawa and Kameoka, 1990). Rhizomes of this plant are the main site for biosynthesis of Glycyrrhizin and Glycyrrhetinic acid (Fiore et al., 2008; Hayashi and Sudo, 2009). The importance of this

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https://doi.org/10.1016/j.funbio.2019.02.003
1878-6146/

plant has increased in the recent past, in modern medicine, due to the activity of Glycyrrhizin against hepatocellular carcinogenesis and prostate cancers (Ikeda et al., 2006; Thirugnanam et al., 2008). The propagation of this plant occurs mostly by vegetative means as the germination rate of seeds is very low (Thirugnanam et al., 2008). The medicinal value of *Glycyrrhiza* and renewed interest in glycyrrhizin has led to its increased demand. To meet this demand, it is important to understand the biology of this plant including the microbiome associated with it so that indigenous microbial strains could be used for its sustainable cultivation and increased productivity. In this study, a comprehensive effort was made to understand the endophytic fungal microbiome associated with *G. glabra* and investigate its bioactive potential for plant growth promotion.

2. Materials and methods

2.1. Study site and sampling

The *G. glabra* plants were collected from 4 different locations *viz*. Jammu (32° 43′N; 74°50′E), Yarikha (34°5′N; 74°47′E), Bonera (33°53′N; 74°55′E), and Solan (30°92′N; 77°15′E) (Fig. 1). The first three locations are the institutional farms of CSIR-IIIM in J&K state, while as the fourth location is the farm of Himachal Pradesh Agricultural University, Himachal Pradesh, India. These locations are geographically different from each another; Jammu (Loc. 1) is humid subtropical, Yarikha (Loc. 2) and Bonera (Loc. 3) are temperate whereas Solan (Loc. 4) has a sub-temperate climate. The selected sites are located at an altitude of 327 m, 2650 m, 1630 m and 1502 m, with an annual precipitation of 1100 mm, 1049 mm, 556.5 mm and 1413 mm, respectively. The healthy plants were collected from each location in the month of Mar/Apr, transported to the laboratory and stored at 4 °C until processed.

2.2. Isolation and cultivation of endophytic fungi

Endophytic fungi were isolated from leaves, stems and roots (rhizome) of *G. glabra* using a previously described protocol (Qadri et al., 2014). The plant material was surface sterilized before plating on media. The effectiveness of the surface sterilization was tested by spreading 1 ml of final wash on potato dextrose agar (PDA) and nutrient agar (NA). The tissues were cut into smaller segments of around 0.5 cm (approx.) and plated on potato dextrose agar, malt extract agar, water agar and Kenknight-Munaier's medium (dextrose 1 g, KH_2PO_4 0.1 g, $NaNO_3$ 0.1 g, KCl 0.1 g, $MgSO_4.7H_2O$ 0.1 g, agar 15.0 g, distilled water 1000 ml, pH 7.2), incubated at 25 °C for 3–4 weeks and observed for any fungal growth. Pure cultures were then maintained on PDA and preserved in 50 % glycerol at −80°. All the media and chemicals were obtained from HiMedia (India). The cultures were submitted to the institutional microbial repository.

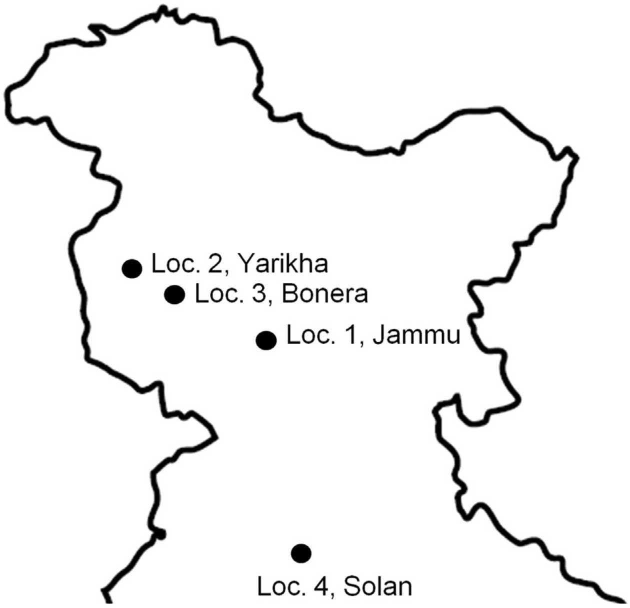

Fig. 1. Geographical map showing the location of the study sites, where Loc. 1 is Jammu (32° 43′N; 74°50′E; Altitude 327 m), Loc. 2 is Yarikha (34°5′N; 74°47′E; Altitude: 2650 m), Loc. 3 is Bonera (33°53′N; 74°55′E; Altitude 1630 m), and Loc. 4 is Solan (30°92′N; 77°15′E; Altitude: 1502 m). The map was modified from Google Maps, Map data ©2018 Google, and modified with Microsoft Office PowerPoint 2007.

The microscopic slides of each endophytic fungus were prepared by staining with lactophenol-cotton-blue (Kiffer and Morelet, 2000) and were examined under light microscope (Olympus BX51, Japan). Based on morphological characteristics, the endophytic isolates obtained were grouped into different morphotypes; and representative isolates of each phylotype was subjected to ITS-based molecular characterization.

2.3. Genomic DNA extraction, acquisition of ITS sequence and phylogenetic analysis

The genomic DNA of the endophytic strains was isolated using a previously described protocol (Wani et al., 2016). The fungal internal transcribed spacer (ITS) (ITS1-5.8S-ITS2) of nuclear ribosomal DNA sequences was amplified by using universal primers, ITS5 (5′-GGAAGTAAAAGTCGTAACAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The sequence of the ITS of rDNA of the isolated strains were submitted to GenBank, and the accession numbers of the sequences (KY419531 to KY419566, KU168142 and KU168143) are given in Table 1. Phylogenetic analyses of the endophytes were carried out by the acquisition of the ITS1-5.8S-ITS2 ribosomal gene sequencing. A threshold of 98 % sequence similarity was used for species level identification. The phylogenetic analyses were conducted in MEGA6.0 (Tamura et al., 2013).

2.4. Diversity analyses

The relative abundance is calculated as the number of isolates of one species divided by total number of isolates of all species and species richness is calculated as the total number of species recovered. Comparison of endophytic diversity analysis at four different locations was carried out using PAST (Version 3.04) software (Hammer et al., 2001). The correlation of endophytic diversity between locations and media used for isolation was analysed using principal component analysis (PCA). The axis values showed the percentage variance of each accessed fungal community. Similarity clustering was also performed using PAST program (Hammer et al., 2001).

2.5. Antimicrobial activity of endophytic fungal extracts

All the 38 endophytic fungi strains were subjected to antimicrobial assay that allows rapid but qualitative selection of the bioactive isolates (Arora et al., 2016). The test organisms used for antimicrobial activity included Gram positive bacteria *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* IIIM 25, Gram negative bacteria *Escherichia coli* ATCC 25922, and fungal pathogen *Candida albicans* MTCC 4748. Each plate had a set of controls as well, a column with a broad-spectrum antibiotic (ciprofloxacin/nystatin) as positive controls, a column without the test extract, and one

Table 1
A summary of forty-nine different OTU's representing the community of fungal endophytes of *Glycyrrhiza glabra* Linn. The table presents the ITS genotypes, GenBank accession numbers, sequence similarity with the closest species and relative abundance for each OTU.

ITS Code	GenBank Acc. no. of the ITS sequence	Species with closest match in GenBank (Acc. no.)	Sequence similarity (%)	Relative abundance
ITS1	KY419541	*Alternaria alternata* (KF039678.1)	99	1.88
ITS2	KY911966	*Alternaria alternata* (KP003824.1)	100	1.13
ITS3	KY419556	*Alternaria brassicae* (JF439438.1)	99	1.50
ITS4	KY419563	*Alternaria burnsii* (KR604843.1)	99	0.75
ITS5	KY419560	*Alternaria porri* (JF422725.1)	99	1.13
ITS6	KY419555	*Alternaria* sp. (KC178622.1)	99	1.50
ITS7	KY419558	*Alternaria tenuissima* (KP278184.1)	99	1.13
ITS8	KY419561	*Aspergillus flavus* (KM285408.1)	99	0.75
ITS9	KY419564	*Aspergillus terreus* (KP143765.1)	100	0.38
ITS10	KY419566	*Bionectria* sp. (GU973628.1)	99	0.38
ITS11	KY419542	*Botryosphaeria dothidea* (KF752588.1)	100	2.63
ITS12	KY419551	*Botrytis cinerea* (KT723007.1)	99	2.26
ITS13	KY419562	*Cladosporium cladosporioides* (HM992526.1)	100	0.38
ITS14	MF039691	*Cladosporium cladosporioides* (LN713977.1)	100	0.38
ITS15	KY419557	*Cladosporium tenuissimum* (AJ300331.1)	99	1.13
ITS16	KY911967	*Colletotrichum gloeosporioides* (JF796301.1)	95	1.13
ITS17	KY419538	*Colletotrichum gloeosporioides* (JX402167.1)	97	1.50
ITS18	KY911968	*Colletotrichum gloeosporioides* (KJ676453.1)	99	0.75
ITS19	KY419543	*Curvularia aeria* (KP131939.1)	99	2.63
ITS20	KY419533	*Diaporthe cotoneastri* (KJ609008.1)	98	7.14
ITS21	KU168142	*Diaporthe terebinthifolii* (NR111862.1)	99	2.63
ITS22	KY419547	*Didymella bryoniae* (FN394714.1)	99	2.26
ITS23	KY419550	*Fusarium avenaceum* (KT963799.1)	99	2.26
ITS24	KY419552	*Fusarium brachygibbosum* (KJ541485.1)	99	1.88
ITS25	KY419559	*Fusarium equiseti* (KR094440.1)	99	1.13
ITS26	KY419537	*Fusarium incarnatum* (KF255436.1)	100	3.38
ITS27	KY419535	*Fusarium oxysporum* (JN400710.1)	100	1.88
ITS28	KY911969	*Fusarium oxysporum* (JN116592.1)	97	1.50
ITS29	KY911970	*Fusarium oxysporum* (KC428403.1)	99	1.13
ITS30	KY419545	*Fusarium solani* (FJ345352.1)	100	1.50
ITS31	KY911971	*Fusarium solani* (JQ625577.1)	98	0.75
ITS32	KY419553	*Lasiodiplodia pseudotheobromae* (KM006451.1)	99	1.50
ITS33	KY419546	*Lasiodiplodia theobromae* (KM357551.1)	99	2.26
ITS34	KY419548	*Macrophomina phaseolina* (JQ676193.1)	100	2.26
ITS35	KY419544	*Mucor circinelloides* (KT583214.1)	99	2.63
ITS36	KY419554	*Mucor hiemalis* (JN206143.1)	99	1.50
ITS37	KY419536	*Phoma exigua* (EU343168.1)	99	4.51
ITS38	KY419532	*Phoma macrostoma* (DQ474112.1)	100	4.89
ITS39	KY911974	*Phoma macrostoma* (LN714588.1)	98	3.01
ITS40	KY419531	*Phoma* sp. (JX045785.1)	100	4.51
ITS41	KY949628	*Phoma* sp. (JX045785.1)	99	3.76
ITS42	KY911972	*Phoma* sp. (KF525844.1)	93	3.76
ITS43	KY419534	*Phomopsis* sp. (KJ463520.1)	99	4.89
ITS44	KY419540	*Rhizoctonia* sp. (KM065559.1)	98	2.26
ITS45	KY911975	*Rhizoctonia* sp. (KM065559.1)	99	1.13
ITS46	KY419549	*Rhizopus oryzae* (KJ417559.1)	99	2.26
ITS47	KU168143	*Stagonosporopsis cucurbitacearum* (KM216012.1)	99	0.38
ITS48	KY419539	*Talaromyces verruculosus* (KP059103.1)	100	3.38
ITS49	KY419565	*Xylaria* sp. (JQ862667.1)	99	0.38

column without the relevant test organism. Ciprofloxacin was obtained from HiMedia and Nystatin from Sigma–Aldrich.

2.6. *Assessment of antifungal potential by dual-culture assay*

Endophytes, representing each taxa of endophytic cultures obtained were investigated for their antagonistic activity against common fungal phytopathogens, to explore them as potential biocontrol agents using the dual culture assay (Wani et al., 2016). The test pathogens used were *Fusarium oxysporum* MTCC 1755, *Colletotrichum capsici* MTCC 2071, *Geotrichum candidum* MTCC 3993, *Sclerotinia* sp. MTCC 7114, and *Aspergillus fumigatus* MTCC 343. The data were obtained from three individual experiments.

2.7. *Biochemical characterization of endophytes*

Endophytic strains were analyzed for various biochemical activities, including hydrolytic enzymes and plant growth promoting activities. The endophytes were screened for the hydrolytic enzymes like amylase, protease and lipase activity was assessed using the protocol described by Mefteh (Mefteh et al., 2017). The endophytes were screened for siderophore production assay using Chrome-azurol S agar medium (Andrews et al., 2016) and indole acetic acid (IAA) production (Wani et al., 2016).

2.8. *Effect of endophytes on plant growth secondary metabolites of* G. glabra

G. glabra plants were grown in flasks containing Murashige and Skoog (MS) medium for 15 d in tissue culture conditions. Equal sized fungal plugs from 5 d old endophytic fungal culture was placed on MS medium a centimetre away from the plant and monitored for two weeks for any pathogenicity. Increase in the number of shoots, leaves, and root mass were taken as the growth parameters. To measure phenolic and flavonoid content, the extracts were prepared from harvested plants of endophyte treated and control plants. Briefly, 1 g of tissue was crushed using liquid nitrogen followed by adding 5 ml of 80 % methanol at 37 °C by continuous stirring for 8 h. This was repeated thrice; extracts were pooled, filtered, and dried using rotary vacuum concentrator and lyophilized. The lyophilized extracts were finally dissolved in methanol and used for measurement of total phenolic and flavonoid content. Total phenolic content was measured using the Folin–Ciocalteu reagent method (Singleton et al., 1999). The total phenolic content was expressed as mg of gallic acid equivalent (GAE) per gram of dry weight of the sample. Total flavonoid content was determined using the aluminium chloride colorimetric method (Akkol et al., 2008). The flavonoid content was calculated from the calibration curve and expressed as quercetin equivalents (mg of QUE per gram of dry weight).

3. Results

3.1. *Molecular phylogeny and diversity of endophytic fungi associated with* G. glabra

A total of 266 fungal endophytic isolates were recovered from 1019 tissue segments. These isolates were grouped into 100 morphotypes based on phenotypic characters like growth pattern, colony texture, colony colour etc (Supplementary Fig. S1). Representative strains from all 100 morphotypes were subjected to molecular identification based on ITS1-5.8S -ITS2 ribosomal gene sequence analyses (Supplementary Table S1). In total, 49 distinct genotypes, spreading over 21 genera and 38 different taxa, were obtained at a 98 % sequence similarity threshold (Table 1). Isolates whose sequences showed less than 97 % sequence similarity or equal similarity above the threshold with more than one species were not documented up to the species level.

Molecular phylogeny assigned most of the endophytes to Ascomycota with 18 genera, followed by Zygomycota with two genera (*Mucor* and *Rhizopus*) and Basidiomycota with one genus (*Rhizoctonia*) (Fig. 2). The most dominant class was Dothideomycetes followed by Sordariomycetes, Mucoromycetes, Eurotiomycetes, Agaricomycetes, Euascomycetes and Leotiomycetes with species richness of 44.7 %, 31.6 %, 7.9 %, 7.9 %, 2.6 %, 2.6 % and 2.6 %, and relative abundance of 46.6 %, 34.2 %, 6.4 %, 3.4 %, 4.5 %, 2.6 % and 2.3 %, respectively (Supplementary Table S2). Leotiomycetes, Euascomycetes and Agaricomycetes were represented by only one taxa each, *Botrytis cinerea*, *Curvularia aeria* and *Rhizoctonia* sp., respectively. Mucoromycetes (Zygomycota) were represented by three genotypes, two belonging to species of *Mucor* and one to species of *Rhizopus*.

The relative abundance of endophytes was highest at Loc. 1 (40.2 %) followed by Loc. 4 (26.7 %), Loc. 3 (21.8 %), and Loc. 2 (11.3 %). The diversity of endophytes at these four locations as calculated by Simpsons and Shannon indices were 0.86 & 2.24, 0.733 & 1.52, 0.83 & 1.93 and 0.77 & 1.77, for Loc. 1, Loc. 2, Loc. 3 and Loc. 4, respectively (Table 2). Therefore, it indicates that the diversity of endophytes is relatively higher in Loc. 1 followed by Loc. 3, Loc. 4 and Loc. 2. This finding is also depicted by the diversity profile graph (Fig. 3).

The most dominant genus among the endophytic fungal community of the host was *Phoma*. Its species collectively represented 24.43 % of the total isolates (almost 1/4th). Thus, the host has strong tendency to establish symbiotic association with *Phoma* (Dothidiomycete). *Fusarium* was the most dominant among the Sordariomycetes and second dominant overall with a relative abundance of 15.42 %. Overall, the most dominant taxa were *Phoma* sp., followed by *Phoma macrostoma*, *Diaporthe cotoneastri*, *Phomopsis* sp. and *F. oxysporum*, respectively (Fig. 4). Species of *Phoma* were the most dominant endophytes of *G. glabra* collected from three sites, while as plants collected from the remaining site (Loc. 3, Bonera), harboured *Diaporthe* as the most dominant endophyte. *Fusarium* was the second most dominant genus at two sites (Loc. 1, Jammu and Loc. 4, Solan), whereas it was the third most dominant at one site (Loc. 2, Yarikha). At the Loc. 3, Bonera, *Fusarium* was the second least found endophyte. Further, *Mucor* and *Phomopsis* species were the second most dominant endophytes found at Loc. 2 (Yarikha) and Loc. 3 (Bonera), respectively.

The biodiversity analysis showed that the colonization frequency was higher in above ground tissues (58.6 %) than the below ground tissues (53.2 %) of the plant. The diversity showed a similar trend as a total of 10 endophytic strains, *Fusarium incarnatum*, *Rhizoctonia* sp., *Mucor circinelloides*, *Lasiodiplodia theobromae*, *Macrophomina phaseolina*, *Lasiodiplodia pseudotheobromae*, *Mucor hiemalis*, *Alternaria tenuissima*, *Alternaria porri* and *Alternaria burnsii* were isolated from the underground parts only, while as 16 endophytic strains, *F. oxysporum, Talaromyces verruculosus*, *Alternaria alternata*, *C. aeria*, *Didymella bryoniae*, *B. cinerea*, *Alternaria* sp., *Alternaria brassicae*, *Cladosporium tenuissimum*, *Fusarium equiseti*, *Aspergillus flavus*, *Cladosporium cladosporioides*, *Stagonosporopsis cucurbitacearum*, *Aspergillus terreus*, *Xylaria* sp. and *Bionectria* sp. were recovered from the above ground part of the plant only. Rest of the 12 strains were isolated from both types of tissues (Fig. 5).

3.2. *Principal components analysis (PCA) and similarity analysis*

Multivariate analysis of the fungal endophytes of *G. glabra* from the four different locations on four different media showed that all the endophytes from Loc. 1 are co-related irrespective of the media used (Fig. 6). The endophytes from Loc. 2 and Loc. 4 are also co-related indicating the influence of environment on the recruitment of endophytes by the plant.

Further, clustering analysis also confirmed the results obtained by multivariate analysis. The endophytes isolated from Loc. 1 formed a separate clade irrespective of media used, while as the endophytes from Loc. 2 and Loc. 4 grouped into a single clade. However, the endophytes from Loc. 3 grouped into two clades (Fig. 7).

3.3. *Antimicrobial activities of the endophytic fungi*

Endophytic fungal extracts from each taxa were estimated for their antibacterial and antifungal activities against a panel of test organisms, *S. aureus* ATCC 29213 and *B. cereus* IIIM 25 (both Gram-positive), *E. coli* ATCC 25922 (Gram-negative), and *C. albicans* MTCC 4748 (a fungal pathogen). Ethyl acetate extracts of each taxa were evaluated for their antibacterial and antifungal activities against a panel of test organisms to find potential strains. A total of 21 extracts were found to possess antibacterial and antifungal activity against one or more pathogens (Table 3). Extracts of 11 fungal endophytes *viz*., *F. oxysporum* GG2F47, *S. cucurbitacearum* GG1F1, *M. phaseolina* GG3F18, *P. macrostoma* GG3F49, *Botryosphaeria dothidea* GG3F1, *Colletotrichum gloeosporioides* GG3F20, *Xylaria* sp. GG3F65, *Phoma* sp. GG2F19, *C. aeria* GG3F56, *Phoma exigua* GR4F69 and *Rhizoctonia* sp. GG2F98 and 4 fungal endophytes *viz*., *S. cucurbitacearum* GG1F1, *A. terreus* GG3F16, *Phomopsis* sp. GR4F42 and *B. cinerea* GR5F59 were potentially active against *B. cereus* and *C. albicans*, respectively with IC50 values of less than 50 μg/ml. Similarly, extracts of 7 fungal endophytes *P. exigua* GR4F69, *C. aeria* GG3F56, *Phoma* sp. GG2F19, *B. dothidea* GG3F1, *P. macrostoma* GG3F49, *S. cucurbitacearum* GG1F1 and *A. flavus* GG2F21 were found active against *S. aureus* with IC50 values less than 50 μg/ml. However, only two endophytic extracts *Xylaria* sp. GG3F65 and *P. exigua* GR4F69 were potentially active against *E. coli*. Extracts of *S. cucurbitacearum* GG1F1, was active against both the Gram-positive pathogens *viz. S. aureus* and *B. cereus* as well as the fungal pathogen, *C. albicans*.

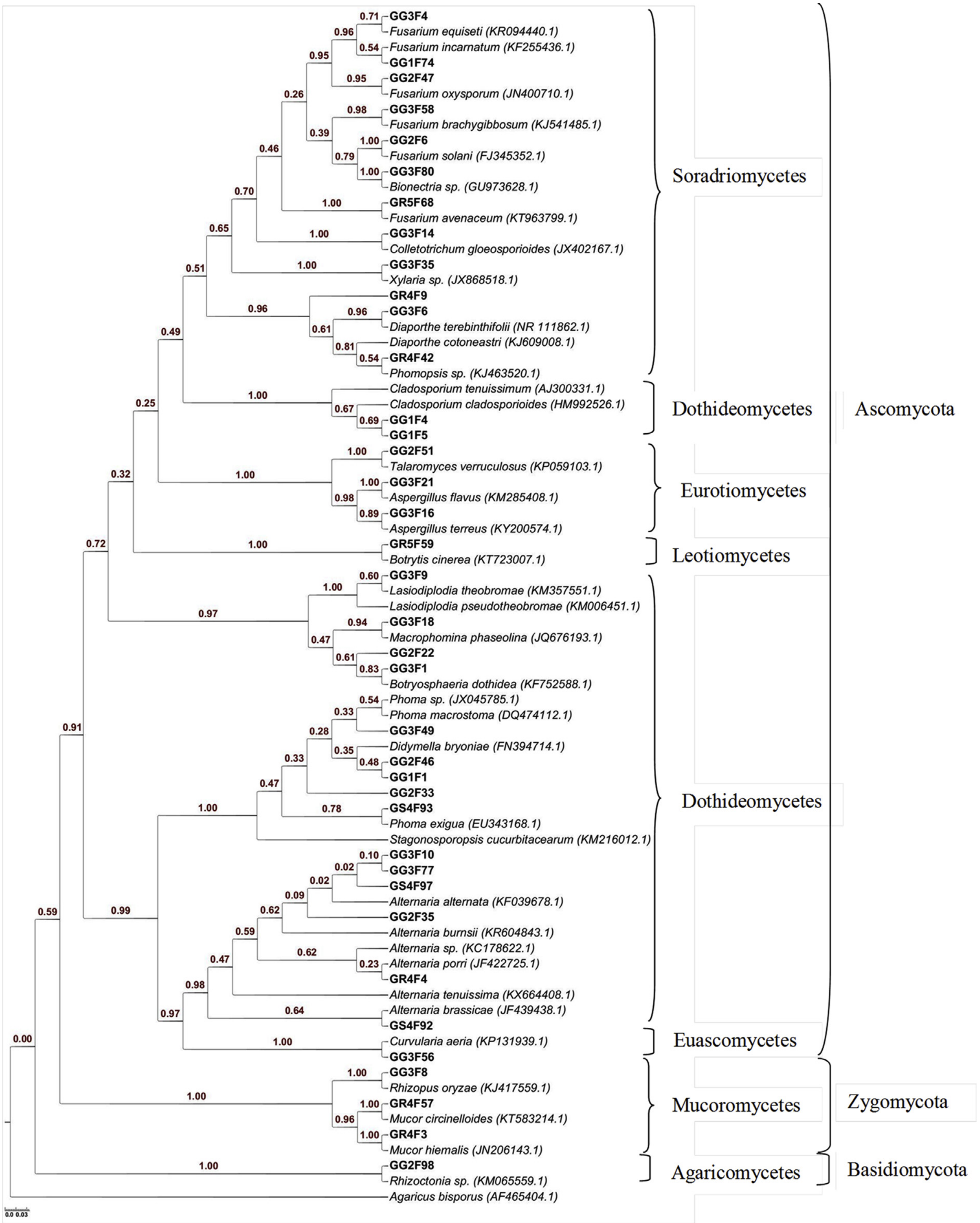

Fig. 2. Phylogeny of endophytes of *G. glabra* using Neighbor-Joining method based on ITS1-5.8S ribosomal gene-ITS2 sequences. The strain numbers of the isolates obtained in this study are presented in bold font. The tree is rooted with *Agaricus bisporus* (an agaricomycetes, AF465404.1). Evolutionary analyses were conducted in MEGA6 (White et al., 1990).

3.4. Antagonistic activity of endophytes against phytopathogens

The fungal endophytes were screened for antagonistic activity against a panel of five fungal pathogens by the *in vitro* dual culture assay to assess their activity against fungal plant pathogens. Three endophytes, *Diaporthe terebinthifolii* GG3F6, *T. verruculosus* GG2F5 and *D. cotoneastri* GR4F9 displayed broad range of antimycotic activity inhibiting more than three pathogens by 50 % or more. The most susceptible pathogens were *F. oxysporum* and *Sclerotinia* sp. which was inhibited more than 50 % by 13 endophytes.

Table 2
Diversity indices of endophytic fungi associated with *G. glabra* at the four locations.

Diversity indices	Loc1 (Jammu; 32° 43′N, 74°50′E)	Loc2 (Yarikha; 34°5′N, 74°47′E)	Loc3 (Bonera; 33°53′N, 74°55′E)	Loc4 (Solan; 30°92′N, 77°15′E)
Genera	15	6	8	8
Individuals	107	30	58	71
Dominance	0.13	0.26	0.16	0.22
Simpson	0.86	0.73	0.83	0.77
Shannon	2.24	1.52	1.93	1.77

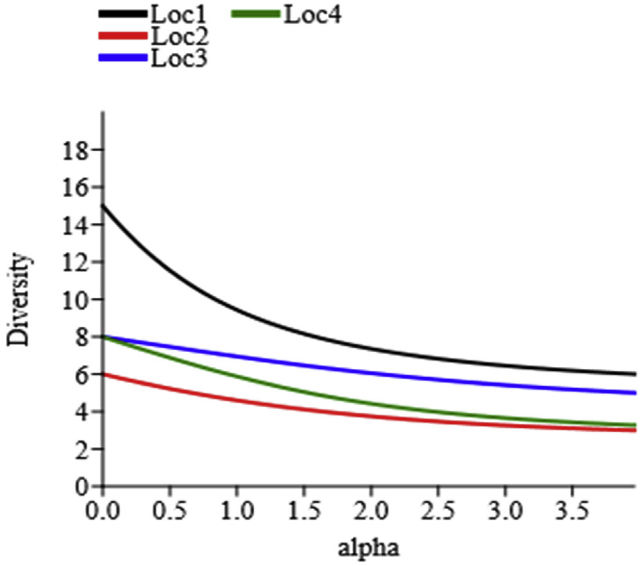

Fig. 3. Diversity analysis of endophytic fungi of *G. glabra* isolated from four different locations, Loc. 1 (Jammu), Loc. 2 (Yarikha), Loc. 3 (Bonera) and Loc. 4 (Solan), using PAST 2.17c software. The diversity of endophytes is relatively higher in Loc1 followed by Loc. 3, Loc. 4 and Loc. 2.

3.5. *Plant growth promoting properties and hydrolytic enzymes*

Screening for siderophore production was done using CAS agar plates and it was found that 20 among the 38 selected (one from each taxa) endophytes produced clear orange halos indicating siderophore production (Table 4). All the endophytic taxa were positive for IAA production under axenic conditions. However, out of 38 endophytic strains, 16 strains were observed to produce IAA more than 50 mg/L, while seven endophytic strains *viz., M. hiemalis* GR4F3, *C. cladosporioides* GG1F5, *C. aeria* GG3F56, *A. tenuissima* GR4F4, *Fusarium solani* GG2F6, *A. terreus* GG3F16 and *F. oxysporum* GG2F47 produced IAA in significantly higher quantities *i.e.*, 341.9, 226.7, 195.8, 149.5, 148.0, 120.0 and 109.5 mg/L, respectively. IAA produced by the two most dominant endophytes ranges from 44.1 to 49.8 mg/l and 15–341.9 mg/l by *Phoma* species and *Fusarium* species, respectively. Representative strains of all the 38 endophytes of *G. glabra* were evaluated for the production of various enzymes like amylase, protease and lipase production. Seven endophytic strains were found to produce amylase, eight endophytes produced protease and twelve endophytes were found to be positive for lipase production.

3.6. *Effect of the endophytic fungi on plant growth*

All the 38 strains of the endophytic taxa were investigated for the pathogenesis test under *in vitro* conditions by co-cultivation with the plant, in order to learn if these endophytes possess the ability to cause disease in the host. It was observed that none of the

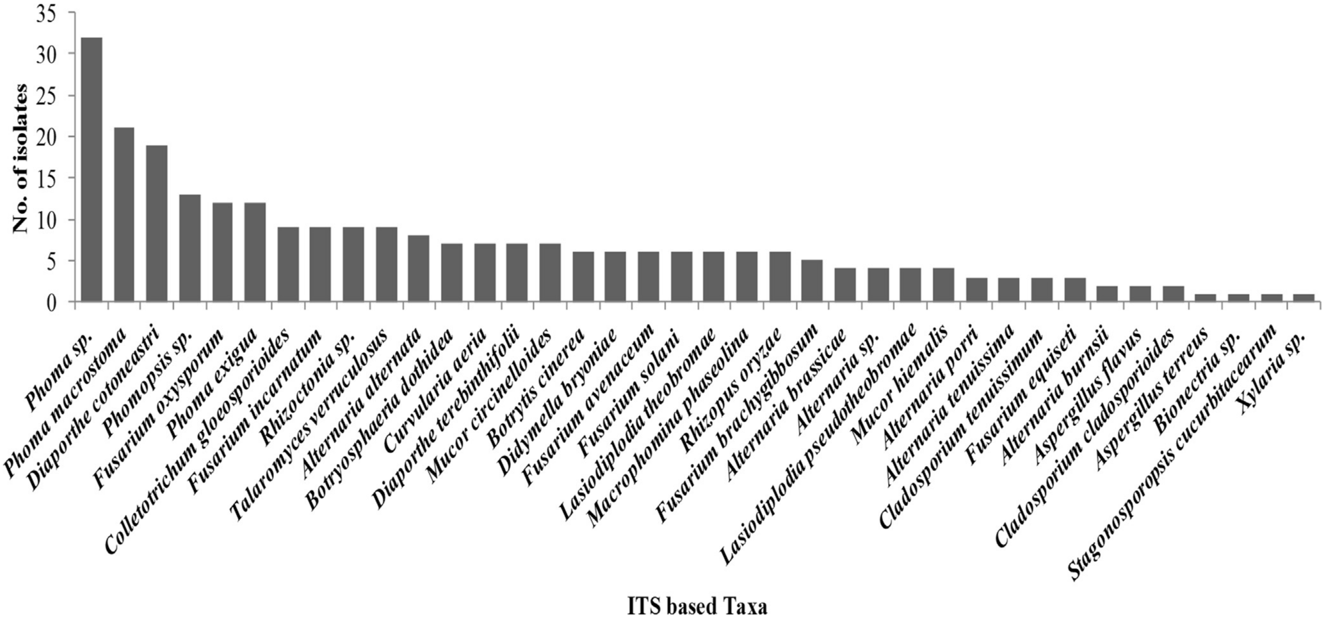

Fig. 4. The relative abundance of 38 different taxa representing the endophytic community of *G. glabra*. The fungal endophytic community of *Glycyrrhiza* was strongly dominated by the species of *Phoma*.

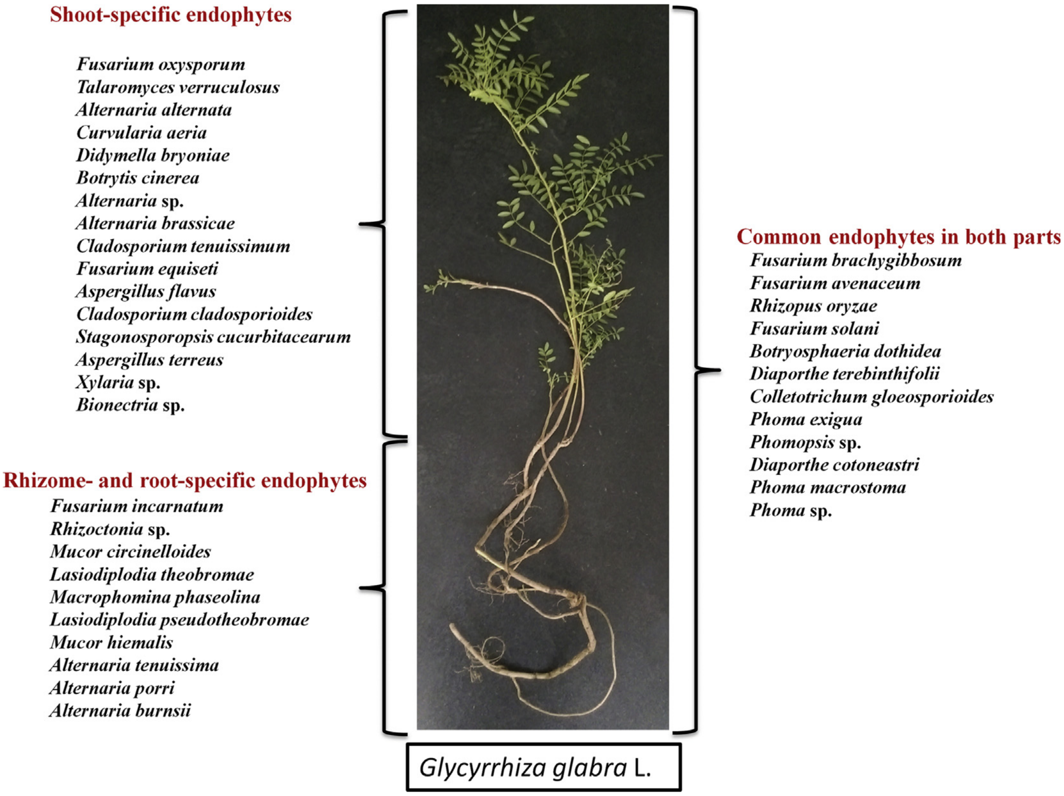

Fig. 5. The biodiversity analyses showed that the colonization frequency was higher in above ground tissues than the below ground tissues of the plant. A total of 10 endophytic taxa were specific to the underground parts, whereas 16 endophytic taxa were specific to the above ground part of the plant. The remaining12 taxa were common to both types of tissues.

endophytes produced any symptoms of disease in the host plant in spite of growing profusely on the tissue culture media. Conversely, three endophytes, *S. cucurbitacearum* GG1F1, *Bionectria* sp. GG3F80 and *A. terreus* GG3F16, increased the root (rhizome) and shoot growth visibly compared to control plants (Fig. 8). Interestingly, all the 38 endophytes taxa had a positive influence on the phenolic and flavonoid content in the host plants (Supplementary Table S3). Six endophytes, *S. cucurbitacearum* GG1F1, *Bionectria* sp. GG3F80, *A. burnsii* GG3F10, *A. porri* GG3F77, *Phomopsis* sp. GR4F42 and *A. terreus* GG3F16, significantly increased the phenolic and flavonoid content by more than 2-fold as compared to the control plants (Fig. 9).

4. Discussion

Due to the ever increasing demand for *G. Glabra* and its metabolites worldwide it is important to explore various possibilities to enhance the production of this medicinal plant and devise methods for its sustainable cultivation. Hence, in this study, we explored the endophytic fungal community of *G. glabra*, grown at

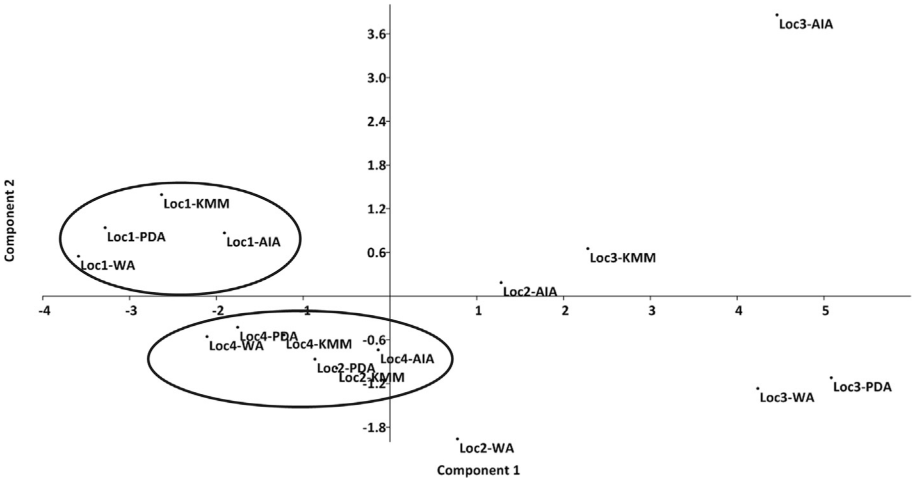

Fig. 6. Multivariate analysis of the isolated fungal endophytes of *G. glabra* from the four different locations Loc. 1, Loc. 2, Loc. 3 and Loc. 4 on four different media (WA, PDA, KMM and AIA): Principal components analysis (PCA) was performed using PAST 2.17c version.

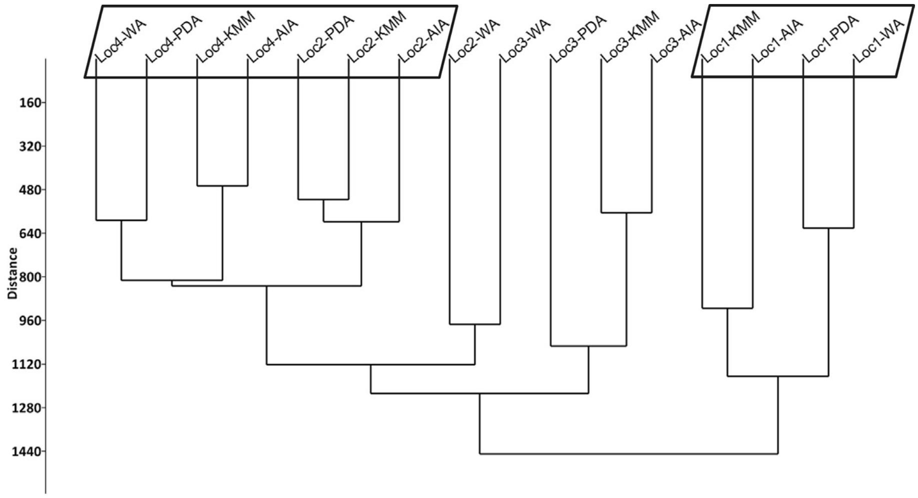

Fig. 7. Clustering of the similarity of endophytic fungal communities from *G. glabra* isolated from four different locations, Loc. 1, Loc. 2, Loc. 3 and Loc. 4 on WA, PDA, KMM and AIA media.

Table 3
Antimicrobial activity of fungal extracts against the bacterial strains, *E. coli* ATCC 25922 (Gram negative), *Bacillus cereus* IIIM 25 & *S. aureus* ATCC 29978 (Gram positive), and *C. albicans* MTCC 4748, a fungal pathogen.

Endophytic Strain	IC$_{50}$ μg/ml			
	Staphylococcus aureus ATCC 29978	*Bacillus cereus* IIIM 25	*Escherichia coli* ATCC 25922	*Candida albicans** MTCC 4748
Alternaria alternata GG2F35	–	–	–	–
Alternaria brassicae GS4F97	–	–	–	–
Alternaria burnsii GG3F10	–	–	–	–
Alternaria porri GG3F77	–	–	–	–
Alternaria sp. GS4F92	–	–	–	–
Alternaria tenuissima GR4F4	–	–	–	–
Aspergillus flavus GG2F21	32.2	–	55.8	–
Aspergillus terreus GG3F16	–	–	–	46.5
Bionectria sp. GG3F80	–	–	–	77.2
Botryosphaeria dothidea GG3F1	45.2	37.2	–	66.3
Botrytis cinerea GR5F59	–	–	–	45.9
Cladosporium cladosporioides GG1F5	–	–	–	–
Cladosporium tenuissimum GG1F4	–	–	–	–
Colletotrichum gloeosporioides GG3F20	–	32.1	–	–
Curvularia aeria GG3F56	43.2	33.2	–	–
Diaporthe cotoneastri GR4F9	–	87.8	–	82.4
Diaporthe terebinthifolii GG3F6	–	86.8	85.2	79.2
Didymella bryoniae GG2F46	–	–	–	–
Fusarium avenaceum GR4F62	–	–	–	–
Fusarium brachygibbosum GG3F58	–	–	65.8	–
Fusarium equiseti GG1F74	–	–	–	–
Fusarium incarnatum GG3F4	53.2	–	–	–
Fusarium oxysporum GG2F47	–	45.2	77.2	–
Fusarium solani GG2F6	–	66.2	52.2	–
Lasiodiplodia pseudotheobromae GG2F22	–	–	–	–
Lasiodiplodia theobromae GG3F9	–	–	–	–
Macrophomina phaseolina GG3F18	66.3	43.1	–	–
Mucor circinelloides GR4F57	–	–	–	–
Mucor hiemalis GR4F3	–	–	–	–
Phoma exigua GR4F69	35.6	32.1	43.2	–
Phoma macrostoma GG3F49	34.5	43.2	77.2	89.2
Phoma sp. GG2F19	25.2	33.2	66.2	72.3
Phomopsis sp. GR4F42	–	–	–	44.2
Rhizoctonia sp. GG2F98	–	34.6	–	–
Rhizopus oryzae GG3F8	–	–	–	–
Stagonosporopsis cucurbitacearum GG1F1	4.1	38.2	–	17.5
Talaromyces verruculosus GG2F51	–	–	–	–
Xylaria sp. GG3F65	–	45.2	33.4	–
Control (Ciprofloxacin/Nystatin*)	0.09	0.04	0.1	1.47

Table 4
Plant growth promoting potential of the endophytes – quantities of indole acetic acid (IAA) produced, antimycotic potential of fungal endophytes against a panel of six common phytopathogens, siderophore production and extracellular enzyme production by the individual endophytes.

Endophytic Strain	IAA (mg/l)	Antimycotic potential[a]	Siderophore	Amylase	Protease	Lipase
Alternaria alternata GG2F35	24.1	−	−	−	−	+
Alternaria brassicae GS4F97	36.7	++	+	+	−	−
Alternaria burnsii GG3F10	59.8	+	−	−	+	−
Alternaria porri GG3F77	109.5	−	−	−	−	−
Alternaria sp. GS4F92	120	−	+	+	−	−
Alternaria tenuissima GR4F4	16.7	−	−	−	−	−
Aspergillus flavus GG2F21	36.6	−	+	−	−	−
Aspergillus terreus GG3F16	20.8	++	+	−	+	−
Bionectria sp. GG3F80	148	++	−	+	−	+
Botryosphaeria dothidea GG3F1	67.2	−	+	−	−	−
Botrytis cinerea GR5F59	45.1	++	−	−	−	−
Cladosporium cladosporioides GG1F5	87.3	+	−	+	−	+
Cladosporium tenuissimum GG1F4	21.1	+	+	−	−	−
Colletotrichum gloeosporioides GG3F20	24.1	+	+	−	−	+
Curvularia aeria GG3F56	37	+++	+	+	+	+
Diaporthe cotoneastri GR4F9	55.9	++	−	−	−	+
Diaporthe terebinthifolii GG3F6	195.8	+	−	+	−	+
Didymella bryoniae GG2F46	26.3	+++	+	−	−	−
Fusarium avenaceum GR4F62	226.7	++	−	−	−	−
Fusarium brachygibbosum GG3F58	24.8	+	+	−	−	−
Fusarium equiseti GG1F74	15	−	−	−	−	−
Fusarium incarnatum GG3F4	49.2	++	−	−	−	−
Fusarium oxysporum GG2F47	55.7	+	+	−	−	−
Fusarium solani GG2F6	341.9	++	+	−	−	−
Lasiodiplodia pseudotheobromae GG2F22	91.2	−	+	−	+	+
Lasiodiplodia theobromae GG3F9	8.3	−	+	+	−	+
Macrophomina phaseolina GG3F18	10	++	+	−	−	−
Mucor circinelloides GR4F57	149.5	−	+	−	−	−
Mucor hiemalis GR4F3	39.1	+	−	−	−	−
Phoma exigua GR4F69	49.8	−	−	−	−	+
Phoma macrostoma GG3F49	44.1	−	+	−	+	+
Phoma sp. GG2F19	45.8	−	+	−	+	+
Phomopsis sp. GR4F42	67.5	−	−	−	−	−
Rhizoctonia sp. GG2F98	7	+	+	−	−	−
Rhizopus oryzae GG3F8	66.7	−	−	−	−	−
Stagonosporopsis cucurbitacearum GG1F1	24.4	+	−	−	−	−
Talaromyces verruculosus GG2F51	38.9	−	−	+	+	−
Xylaria sp. GG3F65	53.8	+++	+	−	−	+

[a] The minus sign indicates none of the pathogens was inhibited by 50 % or more. The plus sign indicate the number of pathogens inhibited by 50 % or more among a total of five pathogens.

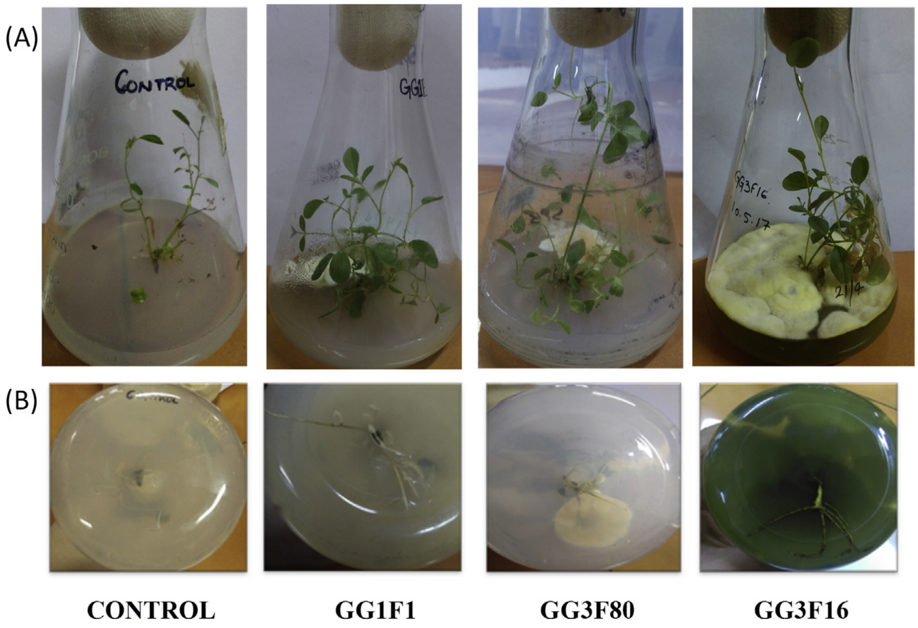

Fig. 8. Co-culture of *G. glabra* plants with endophytes (*Stagonosporopsis cucurbitacearum* GG1F1, *Bionectria* sp. GG3F80 and *Aspergillus terreus* GG3F16) under *in vitro* conditions. (A) Effect of endophytes on shoot growth (B) Effect of endophytes on root/rhizome growth. In the control blank PDA plug and in the endophyte treatments an equal sized plug of individual endophytes were placed on the tissue culture media in proximity with the explants.

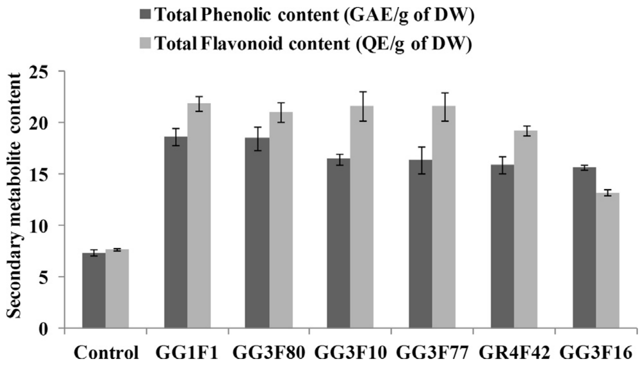

Fig. 9. Effect of endophytes on secondary metabolites (Phenolic and flavonoid) content of *G. glabra*. The endophyte (*Stagonosporopsis cucurbitacearum* GG1F1, *Bionectria* sp. GG3F80, *Alternaria burnsii* GG3F10, *Alternaria porri* GG3F77, *Phomopsis* sp. GR4F42 and *Aspergillus terreus* GG3F16) treatment increased the phenolic and flavonoid content by more than 2-fold as compared to the control plants.

four different locations in the North-Western Himalayas of India, to understand its preferences for endophytes and study their potential for plant-growth promotion. Additionally, an effort was done to conserve this microbial resource for bioprospecting of bioactive secondary metabolites.

Endophytes of *G. glabra* grouped into 38 different taxa encompassing 21 genera, mostly belonging to Ascomycota with rare occurrences of Zygomycota and Basidiomycota. Species of *Phoma* (Dothidiomycete) collectively represented 24.43 % of the total isolates. *Fusarium* was the most dominant genus among the Sodariomycetes and second dominant overall, representing 15.42 % of total isolates. Thus, the host has strong affinity towards establishing symbiotic associations with the fungi belonging to the genera *Phoma* and *Fusarium*. Individually also, *Phoma* dominated the endophytic community of this host at three sites, whereas at the remaining site (Loc. 3, Bonera), *Diaporthe* was the most dominant endophyte. However, the plants from the four sites also showed some significant variations in the community structure of their endophytes. Species of *Diaporthe*/*Phomopsis* were also among the dominant endophytes of the host. In a similar study on *Crocus sativus* from the same area (Kashmir valley), the host was found to possess the dark septate endophytes, *Phialophora mustea* and *Cadophora malorum* as the most dominant endophytes (Wani et al., 2016). These results indicate preferential recruitment of endophytes by the host despite growing in similar or the same environmental conditions. The mechanism of establishment of symbiotic associations by the host plant with the endophytes needs to be studied in order to understand the preferential plant-microbe interactions and their implications on the growth and development of the host. The species richness was highest at the sub-tropical location (Jammu), followed by the sub-temperate location (Solan) and the temperate locations, (Bonera) and (Yarikha), respectively. The results are in agreement with previous reports that there is high species richness of endophytes in tropical climate relative to that in temperate locations (Arnold and Lutzoni, 2007). Also, the colonization frequency of fungal endophytes was more in the above-ground tissues as compared to under-ground parts of the plant. Thus, tissue specificity was evident among the endophytes of the host. These tissues represent two distinct micro-environments consequently shaping their microbiota differently.

Further, to analyze the correlation between locations and media used for isolation of endophytes, we performed the multivariate analysis of the endophytes and it was observed that the geographical location played an important role in the recruitment of fungal communities in *G. glabra*. It was also supported by cluster analysis that shows a higher similarity between the fungal communities isolated from only one location irrespective of the media used. The endophytes from Loc. 2 (Yarikha) and Loc. 4 (Solan) grouped into a single clade although these locations are most distant among the locations selected, and there is also a big difference in the altitude of these sites, Loc. 2 being a high altitude site. How the endophytic communities of these two sites are closely related among the four sites is not therefore clear. Thus, there might be many other factors that influence the endophytic communities of plants growing at a particular site. The plants from these two sites may have the same origin which could be studied by using genetic and chemical markers. In this study, we explored the culturable endophytic fungal diversity associated with *G. glabra.* The application of culture-independent metagenomic approach for the characterization of whole mycobiome may therefore add to the knowledge and give a better understanding of the microbes associated with this host plant. However, only the culturable endophytes can be explored for plant growth promotion and their application in cultivation of the plant.

Endophytes carry out specific functions in nature thus helping the plant in surviving under stress conditions in specific environments, including biotic stress such as invasion by plant pathogens. Thus, endophytes are expected to produce antimicrobial compounds in nature to inhibit the plant pathogens and also compete with other organisms for survival in their specific niches (Wani et al., 2016). In order to learn if the endophytes of *G. glabra* provide the host any resistance to phytopathogens and if these endophytes can be exploited for the production of antimicrobial compounds, we assessed their activity in dual culture against selected plant pathogens and evaluated their extracts for antimicrobial activity against a panel of pathogens. Extracts from some fungal endophytes, particularly *S. cucurbitacearum* GG1F1, isolated in this study showed potential antimicrobial activity. We selected this organism for isolation of pure compounds which lead to the isolation of two thiodiketopiperazine molecules with potential antimicrobial and biofilm inhibition activities against several human pathogens, particularly *S. aureus* ATCC 29213 and *S. pyogenes* MTCC 442 (Arora et al., 2016). We also reported two new hydroxylated unsaturated fatty acids designated as Diapolic acid A–B along with known compounds xylarolide and phomolide G from the endophytic fungus, *D. terebinthifolii* GG3F6 obtained in this study. Xylarolide was found to exhibit potential cytotoxic activity against the breast cancer cell line T47D with an IC_{50} of 7 μM and moderate antifungal activity against *C. albicans* (Yedukondalu et al., 2016). The dual culture assays showed potent activity of some endophytes against plant pathogens inferring that they may be helpful to the host in the development of resistance to pathogens.

Endophytes also enhance the growth and development of the host by virtue of other biochemical properties (Rodriguez et al., 2009). An important aspect of the endophytes studied here is the production of the growth hormone, IAA. All the endophytic taxa produced IAA, *in vitro*, though in varying concentrations. Phytohormone production by endophytes is probably the best-studied mechanism of plant growth promotion, contributing to the overall growth and development of the plant. IAA is found to be involved in biotic and abiotic stress tolerances while it also plays an important role in colonization of endophytes in the plant tissues (Hilbert et al., 2012). This may be the reason for these endophytes being efficient producers of this plant hormone. More than 50 % of the endophytes were able to produce siderophores, indicating their potential growth promoting properties. Interestingly, four endophytes *viz. F. solani, F. oxysporum,C. gloeosporioides* and *C. aeria*, producing IAA in higher amounts were not able to produce any siderophores. In addition, not all the endophytic taxa produced the hydrolytic enzymes which are believed to play some role in colonization in the host tissue. Among the biochemical characteristics studies, the only common trait of the endophytes of *G. glabra* is the production of IAA indicating that this hormone may have a major role in the host-endophyte interactions. To investigate the effect of these endophytes at higher concentrations on the host (pathogenic or plant growth promotion), we observed that under *in vitro* conditions none of them showed pathogenesis to the *Glycyrrhiza* plant, although the fungi were growing luxuriantly on the tissue culture media in co-culture with the plants. Therefore, none of the endophytes were latent pathogens of the host. This is in confirmation that *G. glabra* is rarely infected by any pathogens (Janke, 2004). In contrast, some endophytes of *G. glabra* plant were found to enhance the production of secondary metabolites by the host, particularly total phenolic and flavonoid content, under *in vitro* conditions. Phenolics, flavonoids and carotenoids are responsible for the antioxidant potential of the plants (Agati et al., 2012; Shahidi and Ambigaipalan, 2015). Therefore, the endophytes associated with *G. glabra* may be involved in enhancing the antioxidant potential of the host plant, thereby increasing its pharmaceutical value. The endophytes, *S. cucurbitacearum* GG1F1, *Bionectria* sp. GG3F80 and *A. terreus* GG3F16 are potential candidates for plant growth promotion of the host, as in addition to enhancement of the secondary metabolites, the endophytes visibly increased the root (rhizome) and shoot growth of the host plants.

In conclusion, this study is the first report of community structure, spacial distribution and biological properties of fungal endophytes associated with *G. glabra,* growing under four different climatic conditions in the North-Western Himalayas. The endophytic mycobiome of *G. glabra* comprises of diverse fungi mainly belonging to Ascomycota. *Phoma* and *Fusarium* species dominate the endophytic community of the host, respectively. The endophytes also displayed differential tissue specificity. The diversity was highest at the sub-tropical Himalayan region followed by subtemperate and temperate regions. Several endophytes possessed plant growth promoting traits whereas none was pathogenic to the plant. Although, all the endophytic taxa increased total host phenolic and flavonoid content under *in vitro* conditions, three endophytes were prominent in enhancing the root (rhizome) as well as shoot growth of the host plant. This study indicates that these endophytes may have an important role to play in the growth and development of the host in nature. Thus, the interaction between these endophytes with the host, *in vitro* and *in vivo,* needs to be studied in detail, which might lead to microbial formulations for the sustainable cultivation and productivity of this plant.

Conflicts of interest

The authors have no conflict of interest to report.

Acknowledgements

This work was supported by the Major Lab Project, MLP1008 of the institute and CSIR 12th FYP project, BSC0117. The first author is supported by the Department of Science and Technology, New Delhi, India through INSPIRE Senior Research Fellowship. We are grateful to Prof. Gary A. Strobel, Montana State University, Bozeman, USA, for critically reviewing this manuscript. This research work is a part of the Ph.D. thesis of the first author. The article bears the institutional Manuscript no. IIIM/2179/2017.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.02.003.

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