

Phylogeny, antimicrobial, antioxidant and enzyme-producing potential of fungal endophytes found in *Viola odorata*

Meenu Katoch¹ · Arshia Singh¹ · Gurpreet Singh¹ · Priya Wazir² · Rajinder Kumar¹

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Abstract *Viola odorata*, a medicinal plant, is traditionally used to treat common cold, congestion and cough. Given its medicinal properties and occurrence in the northwestern Himalayas, we isolated and characterized endophytic fungi from this plant morphologically, microscopically and by internal transcribed spacer-based rDNA sequencing. In total, we isolated 27 morphotypes of endophytes belonging to phyla Ascomycota and Basidiomycota. The roots showed the highest diversity of endophyte as well as fungal dominance, followed by leaves and leaf nodes. The fungal extract of VOR16 (*Fusarium oxysporum*) displayed potent antimicrobial activity against *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Escherichia coli*, with a minimum inhibitory concentration of 0.78, 0.78 and 1.56 µg/mL, respectively, while fungal extract VOLF4 (*Aspergillus* sp.) exhibited promising antioxidant activity (IC₅₀ of 17.4 µg/mL). To identify the components responsible for various bioactivities, we analyzed the content of penicillin G in the extract of bioactive endophytes. The results suggested that the expression of penicillin G under the fermentation conditions applied was too low to display antimicrobial effects. Thus, the activity may be contributed by a different, novel secondary metabolite. The

antioxidant activity of VOLF4 may be attributed to its high content of flavonoids. Of the endophytic fungi assessed, 27% were found to be enzyme producers. The highest zone of clearance was observed in VOLN5 (*Colletotrichum siamense*) for protease production. Only VOR5 (*Fusarium nematophilum*) was found to be a producer of cellulase, glutenase, amylase and protease. In summary, this is the first report of the isolation of endophytes, namely *Fusarium nematophilum*, *Colletotrichum trifolii*, *C. destructivum*, *C. siamense* and *Peniophora* sp., from *V. odorata* and their bioactive and enzyme-producing potential.

Keywords *Viola odorata* · Endophytes · Phylogeny · Flavonoid · Penicillin G · Phenolics

Introduction

Endophytes are organisms, often fungi and bacteria, that reside healthy plant tissues without causing any deleterious effects (Strobel and Daisy 2003), exhibiting a variety of interactions with the host that range from mutualistic to antagonistic (Clay and Holah 1999). They occur in all plants on earth, suggesting that they are involved directly or indirectly in the growth, fitness, ecology and evolution of the plant (Brundrett 2006; Vega et al. 2010). The endophytic community varies plant to plant and is affected by a variety of factors, such as environment, season, cultivar, among others (Kaul et al. 2012; Martins et al. 2016). Endophytes also profoundly influence the community structure and diversity of the host-associated organisms, such as bacteria, nematodes and insects (Omacini et al. 2001). Thus, it is imperative to study the endophytic community of a plant inhabiting a particular ecological niche.

Endophytes are a rich source of bioactive natural products, which are currently used in agriculture, medicine and the

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✉ Meenu Katoch
meenusamiksha@rediffmail.com; mkatoch@iiim.ac.in

¹ Microbial Biotechnology Department, CSIR–Indian Institute of Integrative Medicine, Canal Road, Jammu Tawi, Jammu 180 001, India

² Instrumentation Division, CSIR–Indian Institute of Integrative Medicine, Canal Road, Jammu Tawi, Jammu 180 001, India

pharmaceutical industry (Strobel 2003). In the context of emerging health issues, such as novel/drug-resistant pathogens, among others, investigations into endophytic natural products have been played a pivotal role in the global fight against these ailments (Petersen et al. 2004; Cragg et al. 2009). The search for safer and novel natural products is important and can be achieved by selecting the biological resources from a unique environment of extreme age specific to interesting endemic locations or having ethanobotanical uses (Staniek et al. 2008). The northwestern Himalayas is a region that is endowed with unexplored microbial biodiversity. The forests of Bhaderwah, also referred to as “mini Kashmir”, have not been well studied, and the endophytic population of this region remains largely unexplored. The abundance of natural springs, brooks and mountains in this region influence the plant community.

Viola odorata Linn. (Violaceae), commonly called sweet violet or Banafsha, is a medicinal herb native to Europe, Asia, North America and Australia. In India, it grows in Kashmir, mini Kashmir and other parts of the western Himalayas at altitudes ranging from 1500 to 1800 m a.s.l. It is a hardy and annual/perennial herb/shrub that occurs in wild places exposed to sunlight (Erhatic et al. 2010). In traditional medicine, Banafsha has been used as a cure for a variety of skin and respiratory ailments (common cold, congestion, coughing and sore throat), insomnia, anxiety, low blood pressure, headache and cancer pains (Kapoor 1990; Keville 1991; Duke et al. 2002; Kermani and Soroush 2008; Siddiqi et al. 2012; Hamedy et al. 2013). Active constituents of *V. odorata* have been identified as alkaloid, tannins, phenolics, coumarins, flavonoids, glycoside, saponins, methyl saicylate, mucilage and vitamin C (Siddiqi et al. 2012). The plant has been reported to possess antibacterial, antifungal, antioxidant, antitumor, diuretic, laxative, analgesic, antihypertensive, antidyslipidemic anti-inflammatory, antipyretic, sedative, anthelmintic and mosquito-repellant activities (Khattak et al. 1985; Koochek et al. 2003; Svängård et al. 2004; Amer and Mehlhorn 2006; Colgrave et al. 2008; Vishal et al. 2009; Ebrahimzadeh et al. 2010; Akhbari et al. 2012; Barkatullah Ibrar et al. 2012; Alireza and Ali 2013; Zarrabi et al. 2013).

In the study reported here we focused on the diversity, distribution and phylogeny of the endophytic community associated with *V. odorata* endemic to the forests of Bhaderwah and assessed this community as producers of antimicrobials, antioxidants and industrially important enzymes.

Material and methods

Isolation of endophytes

Plants of *V. odorata* were collected during March–April 2014 from the forests of Bhaderwah (mini Kashmir 33.04°N

75.40°E; 1613 m a.s.l.) and identified based on taxonomic features. For the isolation of endophytes, we first carefully excised and then cleaned the roots, leaf nodes and leaves of the plants according to Strobel and Daisy (2003) and Katoch et al. (2014) with slight modification. Clean tissues were serially sterilized in solutions of 70% ethanol, followed by 1.0% sodium hypochlorite (v/v) and then by 70% ethanol, for 1 min/solution, with two final rinses in sterile deionized water; they were then blotted dry and cut into 1-cm² pieces. The pieces were placed on water and potato dextrose agar (PDA) plates containing streptomycin (250 µg/mL). At the same time, the water used for washing the tissues (100 µL) was also plated on PDA plates to confirm the effectiveness of the surface sterilization protocol. All plates were wrapped in Parafilm and incubated at 25 ± 2 °C, and observed daily. The fringes of fungal mycelia growing out from the tissues were subcultured on new PDA plates. These endophytes were deposited in the RN Chopra Microbial Repository, IIIM.

Identification and phylogenetic evaluation of endophytic fungi

The endophytes were identified based on morpho-cultural characteristics and by internal transcribed spacer (ITS)-based rDNA sequencing. Genomic DNA of the endophytes was extracted from the in vitro grown biomass (Raeder and Broda 1985). The ITS1–5.8S–ITS2 region spanning 500–600 bp was amplified with ITS1 and ITS4 universal primers (White et al. 1990). The PCR assay was run in a total volume of 50 µL containing DNA (1–10 ng), 1× PCR buffer (with 15 mM MgCl₂), each dNTP (200 mM), both primers (10 pmol; Integrated DNA Technologies, Leuven, Belgium) and 1 U *Taq* DNA polymerase (Promega, Madison, WI). Cycling parameters were 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min, with a final extension for 10 min at 72 °C. The PCR product [Electronic Supplementary Material (ESM) Fig. S1] was purified using a gel extraction kit (Qiagen, Hilden, Germany), and the sequencing reaction was set up in a volume containing 10 µL:40–60 ng of purified PCR product, 3.2 pmol forward/reserve primer and Big Dye Terminator sequencing mix 8 µL (v. 3.1; Applied Biosystems, Foster City, CA). Samples were sequenced on an automated sequencing system (Applied Biosystems). Resultant sequences (KX621956–KX621982) were submitted to Genbank and blasted against the nucleotide database using the BLASTn tool of the U.S. National Centre for Biotechnology Information for final identification of endophytes (Altschul et al. 1997). For phylogenetic evaluation, endophytic ITS DNA sequences identified in this study and downloaded sequences of their nearest neighbors were analyzed using the tools of MEGA4 software (Tamura et al. 2007).

Fungal diversity evaluation

To evaluate the endophytic fungal diversity associated with *V. odorata*, we calculated different α and β indices, such as Menhinick's index (D_{mn}), Camargo's index ($1/D_{mn}$) or species richness, Fisher's log series index (α), Simpson's Dominance index (D), Simpson's diversity index (SDI; $1 - D$), the Shannon diversity index (H'), and Margalef's richness (D_{mg}) (Hoffman et al. 2008; Tao et al. 2008; Kusari et al. 2013). The similarity indices for endophytic assemblages among tested tissues were also evaluated using Sorensen's (QS) and Jaccard's (Jc) indices using the equations $QS = 2a/(2a + b + c)$ and $Jc = a/(a + b + c)$, respectively, where a is the number of common fungal species found in endophytic populations of two different tissues, and b and c are the number of endophytic fungal species specific to tissues under comparison (Osono and Mori 2004).

Fermentation and extraction

Endophytes were cultured by inoculating two actively growing fungal blocks in potato dextrose broth (400 mL) and incubating the cultures at 27 ± 2 °C and 180 rpm (New Brunswick Scientific Co., Inc., Enfield, CT). After 10 days, each culture was blended thoroughly in 20% methanol and extracted with one volume of methylene chloride [high-performance liquid chromatography (HPLC) grade]. The process was repeated four times. Extracts were vacuum dried.

Antimicrobial activity

Pathogens, namely *Bacillus subtilis* (MTCC No. 121), *Staphylococcus aureus* (MTCC No. 737), *Salmonella typhimurium* (MTCC No. 98), *Pseudomonas aeruginosa* (MTCC No. 424), *Escherichia coli* (MTCC No. 118), *Klebsiella pneumoniae* (MTCC No. 109) and *Candida albicans* (MTCC No. 183), were procured from the Microbial Type Culture Collection (MTCC), Chandigarh (India). All were grown on Muller Hinton agar, with one exception—*C. albicans* was grown on yeast extract peptone dextrose agar.

The antibacterial activity was studied against the test organism by the agar disc diffusion and microdilution assay (Rios et al. 1988; Devaraju and Sreedharamurthy 2011). Streptomycin and amphotericin B were used as positive controls for bacterial pathogens (both Gram-positive and Gram-negative microorganisms) and for *C. albicans*, respectively.

Bacterial strains were inoculated into Muller Hinton broth (HiMedia Biosciences, Mumbai, India) and incubated at 37 °C in a shaker (200 rpm) for 16 h. The cells were quantified according to McFarland standard turbidity [with $0.5 = 1.5 \times 10^8$ colony forming units (CFU)/mL] and ultimately diluted to 1.5×10^5 CFU/mL for all pathogens.

Agar disc diffusion assay

Discs cut out of 10-day-old endophytic cultures (disc diameter 6 mm) were loaded into wells prepared on Muller Hinton agar plates (diameter 90 mm) containing 20 mL medium. A 100- μ L aliquot of cell suspension (1.5×10^5 CFU/mL) of the test organism was then spread on the agar plates and the plates incubated at 37 °C. Antibacterial activities of endophytes were recorded as the zones of inhibition (ZI, in millimeters) produced on each plate. Endophytes were considered to possess effective antibacterial activity when the ZI was ≥ 18 mm (Guimaraes et al. 2008).

Microdilution assay

Different dilutions (1–100 μ g/mL) were prepared from the stock solutions of fungal extract (10 mg/mL) and antibiotic (1 mg/mL). A 150- μ L aliquot of each dilution of fungal extract and antibiotic was mixed with 50 μ L of media containing 4×10^4 bacterial cells (diluted from the stock solution of 1.5×10^8 CFU/mL). Appropriate negative (0.5% dimethyl sulfoxide) and blank controls (virgin media) were used. A 96-well plate was incubated overnight at 37 °C in a incubator shaker with 150 rpm. Activities were expressed in terms of minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC).

Quantification of penicillin G by liquid chromatography-electrospray ionization–tandem mass spectrometry

Penicillin G in endophytic extracts was quantified by liquid chromatography-mass spectrometry.

Preparation of stock solutions of standard compounds A

A stock solution of penicillin G (1.0 mg/mL) was prepared in HPLC grade methanol. Standard working solutions were then obtained by mixing and making appropriate dilutions of stock solutions using the same solvent. The concentration utilized for the preparation of a five-point calibration curve ranged between 3.12 and 50 ng/mL. The standard solutions were filtered through a 0.1 μ polytetrafluoroethylene disposal filter (Whatman; GE Healthcare Life Sciences, Chicago, IL) and injected directly. The stock and working solutions were stored at 4 °C.

Chromatography and LC-electrospray ionization–tandem MS conditions

The chromatography separation was performed on a Purospher® STAR RP18 endcapped column (50 \times 2.1 mm) packed with 3- μ m particles. The mobile phase consisted 10 mM ammonium formate pH 3.5 (A) and acetonitrile (B). An isocratic (50:50%) elution was used over 3 min. The flow rate was 0.3 mL/min. The injection volume was

10 μL and the column temperature was 25 $^{\circ}\text{C}$. Prior to construction of the calibration curve, three blank injections were run to check the noise level of the system. The calibration equation of penicillin G was obtained by plotting the LC-MS peak area (y) versus the concentration (x , mg/mL) and found as $y = 260.632x + 0$ ($R^2 = 0.999$). The equation showed very good linearity over the range. A precursor ion scan experiment on penicillin G also confirmed the origin of daughter ions from the same molecular ion, thereby confirming their presence in the sample. All quantification protocols were performed in triplicate.

Antioxidant activity and phenolics and flavonoid content analysis

Antioxidant activity

The antioxidant activity of each isolated endophyte was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. A 100- μL aliquot of fungal extract (3.125–200 $\mu\text{g/mL}$) was added to a 96-well plate, followed by the addition of 100 μL of DPPH solution (0.2 mM in HPLC grade ethanol) to each well (both test and control wells) and the plate incubated at room temperature in the dark. After 30 min, absorbance was measured at 517 nm. Ascorbic acid and DMSO were used as the positive and negative control, respectively. The free radical scavenging percentage was calculated. The IC_{50} value (50 % inhibition of activity) was interpolated by plotting a linear regression curve:

Free radical scavenging(%)

$$= \frac{(\text{control absorbance} - \text{extract absorbance})}{(\text{control absorbance})} \times 100$$

Total phenolic content

The total phenolic content in each extract was determined by the Folin–Ciocalteu method as described by Cui et al. (2015) with minor modification. A stock solution of extract (10 mg/mL) was made in methanol, and 100 μL of diluted extract was mixed thoroughly with 500 μL of Folin–Ciocalteu reagent (1:10 diluted with distilled water) and 400 μL of sodium carbonate solution (7.5%). The mixture was incubated in the dark for 30 min at room temperature, following which absorbance was measured at 765 nm using a spectrophotometer. The total phenolic content was calculated using the gallic acid calibration curve from the equation $y = 0.030x + 0.118$ ($R^2 = 0.973$), where y is absorbance and x is concentration ($\mu\text{g/mL}$). Total phenolic content was expressed as micrograms/milligram gallic acid equivalent dry weight.

Total flavonoid content

Total flavonoid content was determined using the aluminum chloride colorimetric method described by Zhao et al. (2014) with slight modification. A 400- μL aliquot of crude extract (10 mg/mL) was mixed with a methanolic $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution and incubated for 5 min in the dark. The absorbance was measured at 430 nm, and total flavonoid content was calculated using the quercetin calibration curve from the equation $y = 0.010x + 0.002$ ($R^2 = 0.995$), where y is absorbance and x is concentration ($\mu\text{g/mL}$). Total flavonoid content was expressed as $\mu\text{g/mg}$ quercetin equivalent dry.

Enzyme-producing potential

The enzyme production potential of endophyte was evaluated by the agar disc diffusion assay (Hankin and Anagnostakis 1975). For each enzyme, 1% specific substrate was added to PDA media (starch for amylase; carboxyl methyl cellulose for cellulase; skimmed milk for protease; gluten for glutenase). For estimating the lipase activity, 98 mL of 50 mM Tris HCl, pH 6.8, and 1.2 g agar was autoclaved. In 2 mL of autoclaved Tris HCl, 300 μL of tributyrin and 30 μL of Tween 20 were added and mixed with the Tris-agar and poured over the plates. Wells were prepared in the plates using a sterilized stainless steel cork borer, and the wells then loaded with a 6-mm-diameter disc containing 10-day-old endophytic cultures. The plates were incubated at 27 ± 2 $^{\circ}\text{C}$ for 72–96 h. For amylase/cellulase, plates were stained with Gram's iodine/Congo red (2%) for 5 min. The enzymatic activity was expressed as the average diameter of the degradation halo (ZC). The experiments were repeated three times.

Statistical analysis

Antimicrobial and antioxidant activity data were assessed with analysis of variance and Tukey's post hoc analysis using GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA).

Results

Endophytic fungi associated with *V. odorata*

Twenty-seven endophytes from ten different genera were isolated from *V. odorata* (Table 1). The maximum number of endophytes (14 isolates) was found in roots followed by leaves (7 isolates) and leaf nodes (6 isolates). The majority of the endophytic fungi belonged to phylum Ascomycota, with the exception VOLF5 (*Peniophora* sp.), which belongs to Basidiomycota. These endophytes belong to four different classes: Sordariomycetes (59.25%), Eurotiomycetes

Table 1 Fungal endophytes isolated from tissues of *Viola odorata* and their closest affiliation

Endophyte ID code	Plant tissue	Closest affiliation (accession number)	Identity (%)	E-value ^a	
VOR1	Root	<i>Nigrospora</i> sp. AB693920	99	0.0	
VOR3		<i>Fusarium nematophilum</i> KF498859	99	0.0	
VOR4		<i>Penicillium</i> sp. HZ-3 EU301633	99	0.0	
VOR5		<i>Fusarium nematophilum</i> KF577906	99	0.0	
VOR6		<i>Fusarium solani</i> KM235740	99	0.0	
VOR 7		<i>Colletotrichum destructivum</i> KP259876	100	0.0	
VOR 10		<i>Nectria haematococca</i> JX868649	100	0.0	
VOR11		<i>Colletotrichum truncatum</i> KU498294	99	0.0	
VOR12		<i>Colletotrichum destructivum</i> HQ674658	99	0.0	
VOR 14		<i>Aspergillus awamori</i> LC106113	100	0.0	
VOR15		<i>Fusarium</i> sp. KF472154	99	0.0	
VOR16		<i>Fusarium oxysporum</i> KU872849	99	0.0	
VOR17		<i>Paecilomyces tenuis</i> GQ414523	99	0.0	
VOR18		<i>Fusarium nematophilum</i> KF577885	100	0.0	
VOLN1		Leaf node	<i>Aspergillus</i> sp. KU533844	99	0.0
VOLN2			<i>Daldinia eschscholtzii</i> KJ466979	99	0.0
VOLN3			<i>Aspergillus niger</i> EU440778	100	0.0
VOLN4			<i>Colletotrichum destructivum</i> GU935874	99	0.0
VOLN5	<i>Colletotrichum siamense</i> KP703352		99	0.0	
VOLN7	<i>Penicillium</i> sp. KC871048	99	0.0		
VOLF1	Leaf	<i>Penicillium</i> sp. HZ-3 EU301633	99	0.0	
VOLF2		<i>Colletotrichum trifolii</i> AJ301942	99	0.0	
VOLF3		<i>Colletotrichum destructivum</i> KP259876	100	0.0	
VOLF4		<i>Aspergillus</i> sp. KU533844	99	0.0	
VOLF5		<i>Peniophora</i> sp. KF541333	99	0.0	
VOLF6		<i>Aspergillus japonicus</i> KC128815	99	0.0	
VOLF7		<i>Cladosporium tenuissimum</i> KP701910	100	0.0	

^a Expect value (E): A parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size

(33.25%), Dothideomycetes (3.75%) and Agaricomycetes (3.75%). The most dominant class observed was Sordariomycetes, represented by the orders Trichosphaerales, Hypocreales, Glomerellales and Xylariales, while class Eurotiomycetes was represented by the order Eurotiales. The fungal endophytes belonging to classes Sordariomycetes and Eurotiomycetes were distributed in all plant tissues, whereas those belonging to class Dothideomycetes were found in leaf node tissue and were represented by the order Capnodiales. Endophytes belonging to class Agaricomycetes were restricted to leaves only. *Colletotrichum* spp. (25.9%) showed the highest isolation frequency and were distributed in all plant tissues, followed by *Fusarium* spp. (22.2%), which were restricted to the roots. Endophytes specifically isolated from the leaves were *Colletotrichum trifolii*, *Cladosporium tenuissimum*, *Aspergillus japonicus* and *Peniophora* sp. *Daldinia eschscholtzii*, *Aspergillus niger* and *Colletotrichum siamense* were found to be specific to leaf nodes, and roots harbored *Nigrospora* sp., *Fusarium nematophilum*, *F. solani*, *Colletotrichum truncatum*,

Nectria haematococca, *Aspergillus awamori*, *Fusarium* sp., *F. oxysporum* and *Paecilomyces tenuis*.

Phylogenetic evaluation

The phylogenetic tree provided a more detailed picture of the diverse taxonomic affinities among the endophytic isolates recovered from various tissues of *V. odorata* L., reflecting their evolutionary history (Fig. 1). The endophytic fungi were grouped into four different clusters based on ITS sequences. Each cluster belongs to separate class, i.e. Sordariomycetes, Eurotiomycetes, Dothideomycetes and Agaricomycetes. Of the sequences in cluster I, 43% were related to *Colletotrichum* spp. (VOR7, VOR11, VOR12, VOLN4, VOLN5, VOLF2 and VOLF3) and another 43% are related to *Fusarium* spp. (VOR3, VOR5, VOR6, VOR10, VOR15, VOR16 and VOR18). Other species included in this cluster are *Nigrospora* sp. (VOR1) and *D. eschscholtzii* (VOLN2).



Fig. 1 Phylogenetic position of the endophytic fungal isolates obtained from different tissues of *Viola odorata*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to branches

Cluster II contains a single isolate of *Cladosporium tenuissimum* (VOLF7), while cluster III comprises

endophytes from class Eurotiomycetes, including *Aspergillus* spp. (VOR14, VOLN1, VOLN3, VOLF4 and

VOLF6), *Penicillium* spp. (VOR4, VOLN7 and VOLF1) and *P. tenuis* (VOR17). In cluster IV, there was a single isolate VOLF5 (*Peniophora* sp.) belonging to phylum Basidiomycota.

Fungal diversity analysis

The diversity of the endophytic fungal population isolated from different tissues was evaluated using various α -diversity indices (Table 2). The Simpson Dominance index was highest (0.1667) in leaf node tissue followed by (0.1429) leaf and root tissues (0.1122), whereas Simpson's diversity index was highest in roots (0.8878) followed by leaf (0.8571) and leaf node tissues (0.8333). The Whittaker diversity index was found to be 1.5. Species evenness was more in leaves and leaf nodes than in roots. The Shannon effective number of species was found to be ten for the root, six for the leaf node and seven for leaves. Based on these diversity indices, roots were assessed to host the highest biodiversity, followed by the leaf and leaf node. Higher similarity indices between leaves and leaf nodes depict that the endophytic community of these tissues is more similar (Table 2).

Antimicrobial activity

About 89% of extracts showed antimicrobial activity against the tested pathogens, with the ZI ranging from 20 to 54 mm (Table 3; ESM Fig. S2). In total, 13 (48%), six (22%), 13 (48%), 16 (59%), four (15%) and 15 (55%) extracts exhibited significant antimicrobial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Bacillus subtilis*, respectively. Only three extracts VOLN5

Table 2 Diversity and similarity (Jaccard/Sorenson) indices of endophytic fungi colonizing in tissues of *Viola odorata*

Diversity indices ^a	Plant tissue		
	Root	Leaf node	Leaf
Simpson dominance (<i>D</i>)	0.11	0.17	0.14
Simpson Diversity (1 – <i>D</i>)	0.89	0.83	0.86
Shannon diversity (<i>H</i>)	2.30	1.79	1.94
Menhinick Species richness (<i>D_{mn}</i>)	2.94	2.45	2.64
Margalef species richness (<i>D_{mg}</i>)	3.79	2.79	3.08
Species evenness [e(<i>H/S</i>)]	0.91	1	1
Shannon effective no. of species	10	6	7
Jaccard/Sorenson's indices			
Root	1.0	0.118	0.11
Leaf node	0.06	1.0	0.46
Leaves	0.06	0.3	1.0

^a See Section Fungal diversity evaluation for description of the indices

(*C. siamense*), VOLF4 (*Aspergillus* sp.), VOLF6 (*A. japonicas*) were active against *Candida albicans*, with ZI of 24–32 mm. Extracts of VOLN5 (*C. siamense*) displayed a ZI of 24–54 mm against all the tested human pathogens, including *C. albicans*. Endophytes isolated from root did not display any activity against *S. typhimurium* and *C. albicans*.

In the microdilution assay, 15% of endophytes showed antimicrobial activity with a MIC of >10 $\mu\text{g/mL}$ (Table 3). The VOR16 (*F. oxysporum*) extract showed significant antimicrobial activity against *E. coli*, *K. pneumoniae* and *S. typhimurium*, with a MIC of 0.78, 0.78 and 1.56 $\mu\text{g/mL}$, respectively, while VOR12 (*Colletotrichum destructivum*) was active against *B. subtilis*. Two extracts, VOR18 (*F. nematophilum*) and VOLN1 (*Aspergillus* sp.) were active against *C. albicans*. Endophytes isolated from leaves did not show any antimicrobial activity against any of the tested pathogen at a MIC of <100 $\mu\text{g/mL}$.

To identify the constituent responsible for antimicrobial activity, we measured the penicillin G content in bioactive endophytic extracts by LC–MS, and the results are shown in ESM Table S1 and ESM Figs. S3 and S4). Among the tested extracts, the highest penicillin content was observed in endophyte VOR18 (45.7 ng/mg) followed by VOR16 (42.068 ng/mg), VOLN1 (22.3 ng/mg) and VOR12 (not detected). The total penicillin G were in the order of VOR18 > VOR16 > VOLN1 (highest to lowest).

Antioxidant activity

Four extracts, namely VOR5 (*F. nematophilum*), VOR16 (*F. oxysporum*), VOLN2 (*D. eschscholtzii*) and VOLF4 (*Aspergillus* sp.) showed antioxidant activity, with IC₅₀ values ranging from 17.8 to 50 $\mu\text{g/mL}$ (Table 4). VOLF4 (*Aspergillus* sp.) extract had the best antioxidant activity (IC₅₀ value 17.8 $\mu\text{g/mL}$), while VOLN2 (*D. eschscholtzii*) extract showed the lowest antioxidant activity (IC₅₀ value 50 $\mu\text{g/mL}$).

Total phenolics and flavonoid content present in endophytic extracts showing significant antioxidant activity were determined spectrophotometrically, and the results are shown in Table 4 (ESM Figs. S5, S6). Among the tested extracts, the highest phenolic content was observed in endophyte VOLN2 (120.95 $\mu\text{g/mg}$) followed by VOLF4 (92.5 $\mu\text{g/mg}$), VOR16 (41.06 $\mu\text{g/mg}$) and VOR5 (19.42 $\mu\text{g/mg}$), respectively. Total flavonoids content were in the order VOLF4 > VOLN2 > VOR16 > VOR5.

Extracellular enzyme production

About 17 (63%) endophytes produced one or more enzyme, with a ZC ranging from 20 to 48 mm (Table 5; EMS Fig. S7). Of the endophytes assessed, 33, 7, 26, 37 and 22% were found to be producers of significant levels of cellulase, lipase,

Table 3 Antimicrobial activity of endophytes isolated from tissues of *Viola odorata* by agar disc diffusion and microdilution assays

Endophyte ID	Antimicrobial activity of endophytes against pathogens from the Microbial Type Culture Collection							
	<i>Salmonella typhimurium</i> (MTCC 98)	<i>Klebsiella pneumoniae</i> (MTCC 109)	<i>Bacillus subtilis</i> (MTCC 121)	<i>Pseudomonas aeruginosa</i> (MTCC 424)	<i>Staphylococcus aureus</i> (MTCC 737)	<i>Escherichia coli</i> (MTCC 118)	<i>Candida albicans</i> (MTCC 183)	
	Inhibition zone (mm)	MIC/MBC ($\mu\text{g/mL}$)	Inhibition zone (mm)	MIC/MBC ($\mu\text{g/mL}$)	Inhibition zone (mm)	MIC/MBC ($\mu\text{g/mL}$)	Inhibition zone (mm)	MIC/MBC ($\mu\text{g/mL}$)
VOR1	-	>100	44	>100	40	>100	-	>100
VOR3	-	>100	-	>100	20	>100	-	>100
VOR4	-	>100	34	>100	32	>100	32	>100
VOR5	-	>100	40	>100	36	>100	-	>100
VOR6	-	>100	32	>100	48	>100	-	>100
VOR7	-	>100	46	>100	40	>100	36	>100
VOR10	-	>100	28	>100	28	>100	30	>100
VOR11	-	>100	-	>100	28	>100	-	>100
VOR12 ^a	-	>100	-	1.56/3.125	-	>100	-	>100
VOR14	-	>100	-	>100	40	>100	-	>100
VOR15	-	>100	50	>100	44	>100	20	>100
VOR16 ^a	-	0.78/1.56	-	>100	22	>100	40	>100
VOR17	-	>100	34	>100	38	>100	24	>100
VOR18 ^a	-	>100	40	>100	30	>100	44	>100
VOLN1 ^a	-	>100	-	>100	-	>100	-	>100
VOLN2	-	25/50	20	>100	20	>100	36	>100
VOLN3	-	>100	46	>100	-	>100	34	>100
VOLN4	-	>100	38	>100	38	>100	28	>100
VOLN5	38	>100	22	>100	34	>100	34	>100
VOLN7	-	>100	50	>100	54	>100	38	>100
VOLF2	-	>100	-	>100	30	>100	-	>100
VOLF3	20	>100	-	>100	-	>100	-	>100
VOLF4	-	>100	40	>100	-	>100	-	>100
VOLF5	-	>100	-	>100	-	>100	-	>100
VOLF6	24	>100	44	>100	50	>100	38	>100
VOLF7	-	>100	-	>100	-	>100	-	>100
Streptomycin	20	0.625	18	0.625	18	0.625	18.7	0.625
Penicillin G	-	0.011 MBC	-	0.046 MBC	25	0.046 MBC	-	0.011 MIC
Amphotericin B	ND	ND	ND	ND	ND	ND	ND	24.6

MIC, Minimum inhibitory concentration; MBC, minimum bactericidal concentration (lowest concentration of an antibacterial agent required to kill a particular bacterium)

-, No activity; ND, not done

^a Values presented in bold are highly significant at $P < 0.05$

Table 4 Anti-oxidant activity and phenolics and flavonoid contents in the extract of endophytes isolated from tissues of *Viola odorata*

Endophyte ID code	Anti-oxidant activity (IC ₅₀ ; µg/mL)	Phenolic content (µg/mg)	Flavonoid content (µg/mg)
VOR1	>200	ND	ND
VOR3	>200	ND	ND
VOR5 ^a	22.7	19.42	6
VOR10	>200	ND	ND
VOR11	>200	ND	ND
VOR12	>200	ND	ND
VOR14	>200	ND	ND
VOR15	>200	ND	ND
VOR16 ^a	44.9	41.06	10.8
VOR17	>200	ND	ND
VOLN1	>200	ND	ND
VOLN2 ^a	50	120.95	27.2
VOLN5	>200	ND	ND
VOLN7	>200	ND	ND
VOLF1	>200	ND	ND
VOLF4 ^a	17.8	92.5	106.4
VOLF5	>200	ND	ND
VOLF6	>200	ND	ND

^a Values presented in bold are highly significant at $P < 0.05$

amylase, protease and glutenase enzymes, respectively. The highest ZC (48 mm) was reported in VOLN5 (*C. siamense*)

Table 5 Enzyme production potential of endophytes isolated from tissues of *Viola odorata*

Endophyte ID code	Enzyme activity (ZC, in mm)				
	Cellulase	Lipase	Amylase	Protease	Glutenase
VOR1	–	–	24	22	–
VOR3	–	–	–	40	30
VOR5	20	–	22	28	24
VOR10	–	–	–	–	20
VOR11	20	–	–	20	–
VOR12	40	–	–	–	50
VOR14	–	–	26	20	–
VOR15	38	–	–	–	–
VOR16	–	–	–	–	–
VOR17	40	–	–	–	–
VOLN1	–	40	–	24	–
VOLN2	–	–	30	–	–
VOLN5	–	–	–	48	–
VOLN7	30	–	–	–	30
VOLF1	–	–	–	32	30
VOLF4	20	–	10	44	–
VOLF5	40	–	24	22	–
VOLF6	40	20	20	–	–

ZC, Average diameter of the degradation halo

–, No activity

for protease production. Of 27 endophytic fungi, only VOR5 (*F. nematophilum*) was found to be a producer of cellulase, glutenase, amylase and protease.

Discussion

Several medicinal plants have been explored for the isolation of endophytic fungi (Kaul et al. 2012). We report here the isolation and characterization of endophytic fungi from tissues of *V. odorata*. There were striking differences in the endophytic community of *V. odorata* from the forests of Bhaderwah (33.04°N 75.40°E) (our study) and that from Himachal Pradesh (India) (31.7082°N, 76.9314°E) (Gautam 2013). In our study, most of the endophytic fungi belong to phylum Ascomycota, with the exception of VOLF5 (*Peniophora* sp.) which belongs to phylum Basidiomycota. The endophytic fungi isolated from Himachal Pradesh (India) belong to the Ascomycota and Zygomycota phyla (Gautam 2013). The endophytes from our study belong to classes Sordariomycetes, Dothideomycetes, Agaricomycetes and Eurotiomycetes, while those from Himachal Pradesh (India) have been reported to belong to classes Dothideomycetes and Saccharomycetes. *Aspergillus* and *Cladosporium* endophytes were common in both studies; however, some endophytes, namely *C. trifolii*, *C. destructivum*, *Peniophora* sp. and *Penicillium* sp., were identified from *V. odorata* in our study for the first time. Of these endophytes, *C. trifolii* was reported for the first time as an endophyte. These differences suggest that ecological parameters significantly influence the community of endophytes present in a plant. Similarly, change in the endophytic assemblages due to altitude has also been observed by Hashizume et al. (2008). These endophytes help the host to adapt to a particular niche (Vega et al. 2010).

It is well known that endophytes help the plant to adapt to its ecological niche. *Fusarium nematophilum*, considered to be a nematode-associated fungus (Nirenberg and Hagedorn 2008), is reported here for the first time from *V. odorata* as an endophyte. Isolation of this endophyte might suggest its involvement in protecting *V. odorata* plants from root galls typical of root-knot nematode injury caused by *Meloidogyne javanica* (Martin 1958).

Further, variation in the endophytic community was also observed in the root, leaf node and leaf tissues of *V. odorata*. Interestingly, some of the endophytes found in one tissue did not colonize in other tissues, suggesting that distinct micro-environments of tissues are responsible for structuring their micro-biota. Thus, tissue specificity was evident among the endophytes of *V. odorata*.

The antimicrobial potential of the endophytes was tested using an agar disc diffusion assay and a micro-dilution assay. Using the agar disc diffusion assay, we found that 89% of extracts were active, with a ZI ranging from 20 to 54 mm. Similar the report of Devaraju and Sreedharamurthy (2011),

we found that Gram-positive bacteria were more susceptible to endophytes than Gram-negative bacteria. In the micro-dilution assay, 15% of endophytes showed antimicrobial activity, with a MIC of >10 $\mu\text{g/mL}$. Similarly, Ramos et al. (2010) reported that extracts of endophytes, such as *Papulaspora immerse* and *Arthrinium arundinis*, inhibited the tested pathogens, with a MIC ranging from 90 to 280 $\mu\text{g/mL}$, while extracts of bacterial endophytes isolated from *Aloe vera* displayed antimicrobial activity with a MIC/MBC of 0.625–20 mg/mL. These values are quite low as compared to those determined in our study (Akinsanya et al. (2015).

Antimicrobial activity of one of its endophytic [VOR16 (*F. oxysporum*)] extract against *K. pneumoniae* (MIC 0.78 $\mu\text{g/mL}$) might support the traditional use (to treat cough) of this medicinal plant by producing secondary metabolites through cross-talk between endophyte and host.

To determine the constituent responsible for antimicrobial activity, penicillin G in extracts of bioactive endophytes was estimated by LC–MS. The penicillin G content in extract of the endophytes ranged from 0 to 45.7 ng/mg. For functional activity, it should range between 275 and 1150 ng/mg (calculated with the MICs of penicillin G against different human pathogens shown in Table 4). This leads to the conclusion that the antimicrobial activity in the endophytes isolated in our study is due to secondary metabolite(s) other than penicillin G.

Compounds like penta-ketide (CR377: 2-methylbutyraldehyde-substituted- α -pyrone) and beauvericin, both isolated from endophytic *Fusarium* spp., have been found to be active against *C. albicans*, and methicillin-resistant *S. aureus*, respectively (Sean and Jon 2000; Wang et al. 2011). In our study, VOR18 (*F. nematophilum*) was found to be active against *C. albicans* but as per natural product library, no molecule is known from this species, suggesting further study is needed to isolate the bioactive molecule(s).

Some endophytes (15%) also exhibited potent antioxidant activity. Similar to Li et al. (2015), we found that *Fusarium* spp. (VOR5 *F. nematophilum*; VOR16 *F. oxysporum*) showed antioxidant activity and that *Aspergillus* sp. (VOLF4) showed the highest antioxidant activity, as reported by Yadav et al. (2014) and Khiralla et al. (2015). Previous researchers found that phenolics and flavonoids were responsible for antioxidant potential (Ravindran et al. 2012); moreover, presence of higher amount of phenolics and flavonoid content is directly related to antioxidant potential (Yadav et al. 2014). In our study, extract VOLF4 had both a higher amount of total flavonoid content and showed strong antioxidant activity, with an IC_{50} of 17.8 $\mu\text{g/mL}$. There was no correlation between phenolics content and antioxidant potential. Similarly, extract VOR5 contained the least amount of phenolic and flavonoid contents but showed moderate antioxidant potential, with an IC_{50} value of 22.7 $\mu\text{g/mL}$, possibly due to involvement of

secondary metabolites other than phenolics and flavonoids, such as nitrous compounds, carotenoids and many unidentified compounds (Velioglu et al. 1998; Mahboubi et al. 2013).

The results of our study and of earlier ones indicate that the endophytes have the capability to produce enzymes (Bischoff et al. 2009; Bezerra et al. 2012). Basically, the production of enzymes from an endophyte is associated with its adaption and adaption of the host to that particular environment. Similarly, the production of cellulase and protease enzymes from VOR5 (*F. nematophilum*) is thought to be responsible for biocontrol potential, while amylases and glutenases may be associated with adaption under stress conditions.

In the study reported here, we isolated novel endophytes, such as *Fusarium nematophilum*, *Collectotrichum trifolii*, *C. destructivum*, *C. siamense* and *Peniophora* sp. from *V. odorata* for the first time. Of the endophytes isolated, *Collectotrichum trifolii* is reported for the first time as an endophyte. It is evident from the study that the roots hosted the highest endophyte biodiversity. The isolated endophytes exhibited antibacterial, antioxidant activity and enzyme-producing potential. Antimicrobial activity may be due to some novel secondary metabolite other than penicillin G, while antioxidant activity may be due to the high flavonoid content. Such studies as the one reported here may facilitate the management of such plants under different geographical locations, with a focus on their ex situ conservation, thereby facilitating bioprospecting of potential bioactive molecules and enzymes from endophytes for industrial purposes.

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Compliance with ethical standards

Disclosure The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

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