World J Microbiol Biotechnol (2016) 32:41
DOI 10.1007/s11274-015-1998-8

ORIGINAL PAPER

Molecular phylogenetics and anti-*Pythium* activity of endophytes from rhizomes of wild ginger congener, *Zingiber zerumbet* Smith

D. Keerthi[1] · R. Aswati Nair[1] · D. Prasath[2]

Received: 30 July 2015 / Accepted: 11 December 2015 / Published online: 11 February 2016
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Abstract *Zingiber zerumbet*, a perennial rhizomatous herb exhibits remarkable disease resistance as well as a wide range of pharmacological activities. Towards characterizing the endophytic population of *Z. zerumbet* rhizomes, experiments were carried out during two different growing seasons viz., early-June of 2013 and late-July of 2014. A total of 34 endophytes were isolated and categorized into 11 morphologically distinct groups. Fungi were observed to predominate bacterial species with colonization frequency values ranging from 12.5 to 50 %. Among the 11 endophyte groups isolated, molecular analyses based on ITS/16S rRNA gene sequences identified seven isolate groups as *Fusarium solani*, two as *F. oxysporum* and one as the bacterium *Rhizobium* spp. Phylogenetic tree clustered the ITS sequences from *Z. zerumbet* endophytes into distinct clades consistent with morphological and sequence analysis. Dual culture assays were carried out to determine antagonistic activity of the isolated endophytes against *Pythium myriotylum*, an economically significant soil-borne phytopathogen of cultivated ginger. Experiments revealed significant *P. myriotylum* growth inhibition by *F. solani* and *F. oxysporum* isolates with percentage of inhibition (PoI) ranging from 45.17 ± 0.29 to 62.2 ± 2.58 with *F. oxysporum* exhibiting higher PoI values against *P. myriotylum*. Using *Zz*EF8 metabolite extract, concentration-dependent *P. myriotylum* hyphal growth inhibition was observed following radial diffusion assays. These observations were confirmed by scanning electron microscopy analysis wherein exposure to *Zz*EF8 metabolite extract induced hyphal deformities. Results indicate *Z. zerumbet* endophytes as promising resources for biologically active compounds and as biocontrol agents for soft rot disease management caused by *Pythium* spp.

Keywords *Zingiber zerumbet* · Endophyte · Soft-rot disease · Bioactivity · *Fusarium* spp.

✉ R. Aswati Nair
aswati@nitc.ac.in

[1] School of Biotechnology, National Institute of Technology Calicut (NITC), Calicut, Kerala, India

[2] Division of Crop Improvement and Biotechnology, Indian Institute of Spices Research (IISR), Calicut, Kerala, India

Introduction

Zingiber zerumbet (Family Zingiberaceae) is a perennial rhizomatous herb found either in cultivated, wild or naturalized states (CABI 2014) throughout Southeast Asia, Pacific and Oceania (Yob et al. 2011) with a wide range of ethnomedicinal uses (Vimala et al. 1999; Tushar et al. 2010; Sulaiman et al. 2010; Yob et al. 2011). Among the sixty-nine constituents identified in essential oil from the rhizome, leaves and flowers of *Z. zerumbet*, the sesquiterpenoid zerumbone is the active principle (Dev 1960; Damodaran and Dev 1968; Ruslay et al. 2007) that contributes to its diverse pharmacological properties (Murakami et al. 2002; Yob et al. 2011; Singh et al. 2012). In plants, asymptomatic endophytic assemblages have been identified as important components of plant micro-ecosystem (Zhang et al. 2006; Aly et al. 2011). Endophytic assemblages in various plant taxa are known to be influenced by geographic/edaphic and environmental factors (Arnold and Herre 2003; Owen and Hundley 2004; Kusari et al. 2013; U'ren et al. 2012; Zimmerman and Vitousek 2012). Endophytes involved in such mutualistic associations have received significant attention due to their importance as novel resources for bioactive secondary

metabolites with potential applications in pharmaceutical, agriculture and food industry (Strobel 2003; Qin et al. 2011; Aly et al. 2011; Gutierrez et al. 2012). Besides being important sources of bioactive compounds, endophytes are also known to promote growth of the host plant (Sturz et al. 1997; Surette et al. 2003; Hasegawa et al. 2006; Meguro et al. 2006; Gibert et al. 2012) by nutrient assimilation and phytohormone production (Tan and Zou 2001) and provide tolerance/resistance to abiotic and biotic stress (Chen et al. 1995; Sturz and Matheson 1996; Hallmann et al. 1997; Buchenauer 1998; Shimizu et al. 2000; Rodriguez and Redman 2008; Hasegawa et al. 2006; Conn et al. 2008).

Endophytes have also been identified as useful biocontrol agents (Kunoh 2002; Backman and Sikora 2008) inhibiting phytopathogenic growth as observed in cotton against pathogenic *Fusarium oxysporum* subsp. *vasinfectum* and in potato against *Verticillium albo-atrum*, *Rhizoctonia solani* (Hallmann et al. 1997) and *Clavibacter michiganensis* subsp. *sepedonicum* (van Buren et al. 1993). Antagonistic activities of endophytes against phytopathogens are mediated by mechanisms that include antibiosis, induced systemic resistance, competition for niches and nutrition or predation and parasitism (Arnold et al. 2003; Schulz and Boyle 2005; Conn et al. 2008; Rodriguez et al. 2009; Aly et al. 2011; White and Bacon 2012). *Z. zerumbet* has been previously documented to exhibit resistance to necrotrophic oomycetous *Pythium* spp., the causative agent of soft-rot disease (Kavitha and Thomas 2007) manifested as water-soaked and putrefied rhizomes. Our previous studies have demonstrated the significant role of zerumbone, the active principle in *Z. zerumbet* in imparting resistance to soft-rot causative *P. myriotylum*. Besides no major diseases have so far been reported in the wild taxa except for reports indicating the taxa serving as a minor host for the spiraled whitefly, *Aleurodicus disperses* and cardamom root grub, *Basilepta fulvicornis* (CABI 2014). Despite the remarkable resistance exhibited by *Z. zerumbet*, obscure information is available on the endophytic micro-biota of the taxon. Hence the present study was undertaken towards (1) bioprospecting the endophytic assemblage of *Z. zerumbet* rhizomes and (2) determining its biological control potential against soft rot causative *P. myriotylum* strain.

Materials and methods

Sample collection and preparation

Healthy *Z. zerumbet* rhizomes devoid of any external lesions were collected from Indian Institute of Spices Research (IISR), Calicut, India. Rhizome samples (5–6 intact rhizomes) were collected at two different times of growing season viz., during early-June of 2013 and late-July of 2014. The collected rhizomes were thoroughly washed under running water for an hour to remove all soil. Rhizomes were surface-sterilized by sequential washes in 20 % sterilisation solution (5 % sodium hypochlorite and 0.01 % Tween 20), twice with 70 % ethanol for one minute followed by 0.1 % mercuric chloride for 8 min. Finally traces of sterilizing agents were removed by washing the tissues with sterile water six times for 5 min each. The water obtained from last wash was plated on potato dextrose agar (PDA) to ensure complete surface sterilization.

Isolation of endophytes

The surface sterilised rhizomes were used for isolation of *Z. zerumbet* endophytes. Rhizome pieces were placed on fresh sterile PDA (pH 6.4) plates supplemented with 60 mg/ml ampicillin and incubated at 25 ± 3 °C for 7 days for growth initiation. Fungal isolates growing out of the rhizome pieces were sub-cultured onto the same medium while the bacterial isolate obtained were grown in Luria–Bertani (LB) medium without the antibiotic. Purified isolates thus obtained were assigned codes and maintained in their vegetative form in PDA/LB plates and as stock cultures in glycerol suspensions (50 % w/v) at −80 °C.

Morphology of *Z. zerumbet* endophytes

Morphological characteristics were determined after incubation for 14 days at 25 ± 3 °C on PDA/LB medium. Designations of colony colors were made by comparing with color charts of Inter-Society Colour Council-National Bureau of Standards (ISCC–NBS) (Kelly 1964). Microscopic morphological characters were determined using bright field trinocular research microscope (Olympus BX51) and included characteristics such as size/shape of conidia and mycelia septation which were used as classic confirmatory characters to identify fungal isolates according to standard taxonomic key (Ainsworth et al. 1973). For bacterial isolate, the following traits were evaluated: color, surface, margin, opacity, gram staining, motility and spore formation.

Colonization frequency (CF %) of endophytes was calculated as: $CF = (N_{col}/N_t) \times 100$, where N_{col} and N_t are the number of segments colonized by each endophyte and the total number of segments observed respectively (Hata and Futai 1995).

DNA extraction and isolation

Genomic DNA was isolated from actively growing mycelium scraped from PDA plates and from LB bacterial culture using modified Cetyltrimethyl ammonium bromide

(CTAB) procedure (Rogers and Bendich 1994). Briefly mycelium ground in liquid nitrogen and the bacterial suspensions were transferred to pre-warmed CTAB buffer and incubated for 1 h at 65 °C. Homogenate was extracted thrice with chloroform- isoamyl alcohol (24:1) and centrifuged at 14,000 rpm for 15 min. The extract was treated with RNase (20 mg/ml) and incubated for 45 min at 37 °C. From the clear RNA free supernatant, DNA was precipitated using two volumes of ice-cold isopropanol and incubated overnight at 4 °C. Precipitated samples were centrifuged and the DNA pellet was rinsed with 70 % (v/v) ethanol, air dried, dissolved in 0.1 M TE (Tris–EDTA; pH 8.0) buffer and stored at −20 °C for further use.

PCR amplification

The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using eukaryotic universal primers, ITS 1 (5′-TCCGTAGGTGAACCTGCGG–3′) and ITS 4 (5′TCCTCCGCTTATTGATATGC–3′). For identification of bacterial isolate, 16S rDNA region was amplified using universal primers, 16SrF (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SrR (5′-GGTTACCTTGTTACGACTT-3′). Reaction mixture contained 10X PCR reaction buffer with 1.5 mM $MgCl_2$, each dNTP at 10 mM concentration, primers at 10 pmol concentration, *Taq* DNA polymerase and 10 ng/µl DNA. Thermo-cycling was done in S1000 Thermal cycler (Bio Rad, USA) and consisted of an initial denaturation step at 94 °C for 3 min, followed by 35 amplification cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final 5 min extension at 72 °C. PCR products were electrophoretically examined in 1.2 % agarose gel and the amplicons were excised and purified using Wizard SV gel and PCR Clean-up System (Promega, WI, USA) following manufacturer's instructions prior to sequencing.

Sequencing and phylogenetic analysis

Sequencing was performed on the Applied Biosystems 3730XL DNA Analyser. Sequence data were screened by visual inspection of chromatograms using Chromas and the primer sequences were removed. Sequences obtained were subjected to homology searches using BLAST algorithm in NCBI database (www.ncbi.nlm.nih.gov). The ITS sequences were subjected to multiple alignment with homologous sequences using CLUSTAL W (Thompson et al. 1994). Phylogenetic relationships of the isolated endophytes were inferred using neighbour-joining (N-J) (Saitou and Nei 1987), maximum parsimony (MP) (Fitch 1971) and maximum likelihood (ML) (Felsenstein 1981) tree making algorithms using Mega 5 software (Tamura et al. 2011). Statistical validation at each node was determined by 1000 bootstrap replicates.

Determination of *P. myriotylum* antagonistic activity

Antagonistic activity of the endophytic strains against the *P. myriotylum* was studied following dual culture assay (Lahlali et al. 2007). Mycelial discs (5 mm diameter) from 7-day old cultures of each fungal endophyte and *P. myriotylum* were placed on opposite sides of the same PDA plate. Control plate consisted of only the *P. myriotylum* discs. Plates were incubated at 25 ± 3 °C for 7 days and radial mycelial growth of each endophyte against the pathogen was recorded. Percentage of inhibition (PoI) was calculated as described by Rahman et al. (2009) as: $PoI = [(R1 - R2)/R1] \times 100$, where R1 and R2 are radii of fungal phytopathogen colony in control plate and test plate respectively. All experiments were conducted in triplicate.

Strain displaying highest anti-*Pythium* activity was inoculated in potato dextrose (PD) medium for 14 days at 25 °C. After fermentation, the mycelial mat was harvested, ground and extracted with absolute dichloromethane (DCM) at room temperature for 24 h. The organic phase was concentrated on rotary evaporator (Heidolph, Germany) in reduced pressure at 42 °C for 30 min. The obtained DCM fractions were subject to bioassays for evaluating anti-*P. myriotylum* activity by disc diffusion method. Briefly mycelial disc (5 mm) from 7-day-old *P. myriotylum* culture grown in PDA was placed on a Whatman No. 4 filter paper disc (10 mm) impregnated with increasing dilutions of extract (1–40 µl) in the center of PDA plates. In control experiments, PDA discs were placed on a filter paper impregnated with DCM. Plates were incubated at 25 °C for 4 days, and percentage inhibition was measured by comparing the mycelial growth in the test plate with that of control plate. Growth inhibition was calculated using the formula: $[I\ \% = (C - T) \times C^{-1}] \times 100$ where I % is the relative inhibition, C is the control radial diameter of *P. myriotylum* hyphae in presence of solvent and T is the radial diameter of *P. myriotylum* hyphae in presence of extract.

Scanning electron microscopy (SEM) analysis

P. myriotylum mycelium grown on polylysine-coated glass cover slips was exposed to 5 µL of metabolite extract from antagonistic endophyte for 2 h. Treated and untreated mycelium was fixed with glutaraldehyde (2.5 % v/v) in 0.1 M phosphate buffer (pH 7.5) for 3 h at 25 °C. Fixation was followed by washing with phosphate buffer (pH 7.5) and dehydration with graded ethanol series (30, 50, 70, 90, 95 and 100 %) for 10 min in each series. The fixed samples were mounted on stubs using double-sided carbon tape and coated with gold using sputter coater system (E-1010 ion sputter, Hitachi) for 30 s at 10–20 Pa vacuum and current

density of 10 mA. SEM images were captured using S06600SEM (Hitachi) at an accelerating voltage of 5 kV.

Results

Isolation and morphological characterization of endophytes

Endophytes totalling 34 were collectively obtained from 10 asymptomatic 15 mm^2 rhizome segments of *Z. zerumbet*. The isolates were found to be dominated by fungi with only one bacterial gram-negative strain. Preliminary grouping according to morphological characteristics categorized the 34 endophytes to 11 groups (Table 1). Among the 11 morphologically distinct *Z. zerumbet* endophytes, ten were fungi and one was a bacterium with CF (%) values ranging from 12.5 to 50 % (Table 1). Two of the fungal groups were observed to form purple mycelium on PDA medium with good growth observed at 25 ± 3 °C (Fig. 1 C, D).

Molecular identification of *Z. zerumbet* endophytes

The amplified ITS/16S rDNA region of endophyte groups were sequenced and homology searches using BLAST algorithm revealed that of the 11 endophyte groups isolated, seven showed ≥96 % similarity to *Fusarium solani*, two isolate groups showed 99 and 100 % similarity to *F. oxysporum* and one designated *Zz*EB1 to the bacterial strain, *Rhizobium* spp. (99 % identity). Sequences with e-value ≥0.0 and ≥96 % identity with the amplified ITS/16S rRNA sequences of *Z. zerumbet* endophytes were used for multiple alignment using CLUSTALX. Phylogenetic analysis revealed two distinct clades representing *F. solani* and *F. oxysporum* respectively (Fig. 2A). Among the *Fusarium* homologous isolates *Zz*EF1–*Zz*EF6, *Zz*EF9 and *Zz*EF10 clustered with *F. solani* while *Zz*EF7 and *Zz*EF8 clustered with *F. oxysporum* ITS sequences in the ML tree (Fig. 2A). The ML based phylogenetic classification was maintained in the trees generated using NJ and ME tree-making algorithms. ML analysis of 6S rRNA sequences from *Rhizobium* to related genera viz., *Azorhizobium* and *Bradyrhizobium* clustered the bacterial endophyte designated *Zz*EB1 with *Rhizobium* spp. (Fig. 2B).

Bioactivity of *Z. zerumbet* endophytes against *P. myriotylum*

Confrontation experiments or dual culture assays revealed limited mycelial growth of *P. myriotylum* in presence of *Z. zerumbet* fungal endophytes with PoI ranging from 7.01 ± 3.31 to 63.28 ± 2.53 % (Table 2). Among the ten fungal isolates, six endophytes designated *Zz*EF2, *Zz*EF3, *Zz*EF5, *Zz*EF6, *Zz*EF9 and *Zz*EF10 identified as *F. solani* were able to inhibit *P. myriotylum* growth by PoI ranging from 45.17 ± 0.29 by *Zz*EF3 to 54.41 ± 3.81 by *Zz*EF4. Endophytes designated *Zz*EF7 and *Zz*EF8, representing *F. oxysporum* isolates yielded high PoI values against *P. myriotylum* of 63.28 ± 2.53 and 62.2 ± 2.58 respectively (Table 2). To further evaluate inhibitory effect of endophyte *Zz*EF8, metabolite was extracted from mycelium with DCM which was observed to inhibit *P. myriotylum* hyphal growth with the antagonistic activity observed to be concentration dependent (Fig. 3A). SEM analysis was carried out to examine the effect of *Zz*EF8 metabolite extract on surface topography of *P. myriotylum* hyphae. Shrivelling and distortion of hyphae accompanied by collapse at various sites was observed (Fig. 3C) compared to the linear hyphae with homogenous width as seen in the control (Fig. 3B).

Discussion

The ubiquitous but selective colonization of endophytes in plants prompted us to undertake the present novel attempt towards characterization of endophytes from *Z. zerumbet* rhizomes that has a broad spectrum of pharmacological activities besides exhibiting remarkable disease resistance (Kavitha and Thomas 2007; Aswati and Thomas 2007; CABI 2014). Except for *Z. officinale* (Jasim et al. 2014), no *Zingiber* taxa have been investigated so far for their endophytic assemblage despite their ethnomedicinal significance. Higher endophyte density is reported in many taxa in the roots/rhizomes and decreases acropetally (McInroy and Kloepper 1995; Quadt-Hallmann et al. 1997). The low rate of colonization constituting 11 endophyte groups isolated in the present study may be attributed to the spectrum of secondary metabolites with anti-microbial properties produced in the rhizomes (Yob et al. 2011; Singh et al. 2012; Ruslay et al. 2007). Similar low endophyte frequency was also reported in other medicinal plants with 9 isolates from *Dioscorea zingiberensis* rhizomes (Xu et al. 2008), 16 isolates each from *Coffea robusta* (Sette et al. 2006) and *Argyrosomus argentatus* (Liu et al. 2005). Such low endophyte densities obtained could also be attributed to the concentration and incubation time of disinfection process (Hallmann et al. 1997).

Based on morphological and phylogenetic analysis of ITS/16S rRNA sequences, the most frequent fungal endophyte colonizing *Z. zerumbet* was identified as *Fusarium* spp., which also happens to be a major pathogen affecting ginger (*Z. officinale*) productivity. Despite the pathogenicity of *Fusarium* spp., it has been reported to exist in symbiotic association in many plant taxa (Shiono et al. 2007a, b; Kaur et al. 2010) and is used as a biocontrol agent against various phytopathogens (Ghini et al. 2000;

Table 1 Endophyte groups identified from *Z. zerumbet* rhizomes based on morphological characteristics and molecular analyses based on ITS/16S rRNA gene sequences

Isolate code	Morphological characteristics	Shape of conidiospores	CF (%)	Closest homology in NCBI	Accession number	e-value	ITS identity (%)
ZzEF1	Compact colony having fine ends growing in concentric rings	Elliptical to olivary shaped macroconidio spores	12.5	*F. solani*	HQ265424	1e-152	98
ZzEF2	Compact colony growing in concentric rings	Elliptical to olivary shaped macroconidiospores	12.5	*F. solani*	LN828110	7e-154	99
ZzEF3	Compact white colony	Falcate macroconidio spores	25	*F. solani*	KJ528882	2e-133	99
ZzEF4	Compact white with abundant mycelia	Falcate macroconidio spores	25	*F. solani*	KM066557	3e-162	99
ZzEF5	Compact raised white colony	Elliptical or olivary macroconidiospores	12.5	*F. solani*	KM066557	3e-142	96
ZzEF6	Compact white colony	Falcate macroconidio spores	12.5	*F. solani*	FR691777	4e-141	99
ZzEF7	White colony initially, later turned to deep red upon maturity	Olivary or elliptical conidiospores	50	*F. oxysporum*	KJ439211	6e-129	99
ZzEF8	White dense colony initially, turned to vivid reddish orange and later to very deep red upon maturity	Olivary or elliptical conidiospores	50	*F. oxysporum*	KC304806	1e-130	99
ZzEF9	White compact colony growing in concentric rings	Ovoid to barrel shaped spores	12.5	*F. solani*	KM519190	5e-160	100
ZzEF10	White compact colony growing in concentric rings	Ovoid to barrel shaped spores	12.5	*F. solani*	KM519190	5e-160	100
ZzEB1	Smooth, round pale yellow colony, Gram negative, non-motile	No endospores observed	50	*Rhizobium* spp.	KF922663	0.0	99

Morphological features, colonization frequency (CF; %) and results of homology searches using BLAST algorithm are indicated

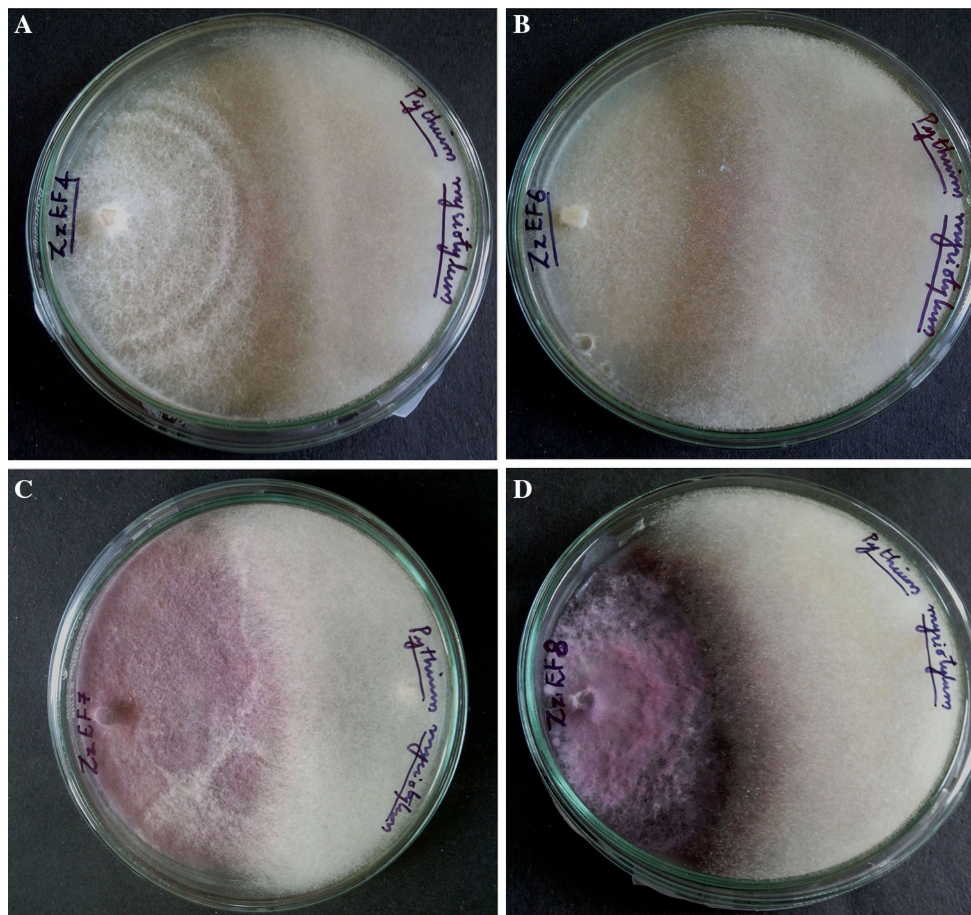

Fig. 1 Anti-*Pythium* activity determined by dual-culture bioassay of four representative endophytes isolated from *Z. zerumbet* rhizomes. **A** *Zz*EF4; **B** *Zz*EF6; **C** *Zz*EF7 and **D** *Zz*EF8

Kaur et al. 2010) that includes burrowing nematode and banana weevil (Paparu et al. 2009) of banana, *Fusarium* wilt disease (Nel et al. 2006) of banana and cucumber (Mandeel and Baker 1991). *Fusarium* spp. has been previously reported as endophyte colonizing different medicinal plant taxa with various bioactivities (Shiono et al. 2007a, b) such as *Camptotheca acuminata* (Ding et al. 2013), *Taxus baccata* (Tayung et al. 2011), *T. chinensis* (Deng et al. 2009), *T. celebica* (Chakravarthi et al. 2008), *Juniperus recurva* (Kour et al. 2008) and *Dysoxylum binectariferum* (Mohana Kumara et al. 2012). Earlier studies have reported phytopathogenic species as prevalent endophytes colonizing various plants such as *Colletotrichum* spp. in *Artimisia* spp. (Huang et al. 2009) and *Jatropha curcas*, *Erwinia* spp. in cotton (Misaghi and Donndelinger 1990), *Xanthomonas* spp. in pepper (Bashan et al. 1982) and *Pseudomonas* spp. in olive (Gómez-Lama Cabanás et al. 2014). Accumulating body of evidence suggests that pathogenic endophytes have been horizontally (Rodriguez et al. 2009) or vertically (Cook et al. 2013; Hodgson et al. 2014) transmitted and play an important role in plant defense (Arnold et al. 2003; Jaber and Vidal 2010; Gange et al. 2012) according to tenets of mutualism theory (Arnold et al. 2003; Rodriguez et al. 2009; White and Bacon 2012). Mutualistic existence of endophytes profoundly influence host plant fitness (Brundrett 2006) by contributing towards nutrition/growth and defense with the latter function underpinning the "defensive mutualism" concept (DMC) (Clay 1988; Saikkonen et al. 2010; Panaccione et al. 2014) and provides explanation for widespread occurrence of systemic endophytes in various plant taxa. The bacterial endophytic genera identified from *Z. zerumbet* rhizomes viz., *Rhizobium* spp. have been reported to exist in symbiotic association especially in legumes (Dudeja et al. 2012) and also in various plant taxa (Gutiérrez-Zamora and Martínez-Romero 2001).

Present study also reports for the first time the antagonistic activity of *Fusarium* spp. and other endophyte isolates from *Z. zerumbet* rhizomes against *P. myriotylum*. *Z. zerumbet* endophytes, *Zz*EF7 and *Zz*EF8 identified as *F. oxysporum* exhibited potent anti-*Pythium* activity in dual culture assays and were observed to produce a red-colored

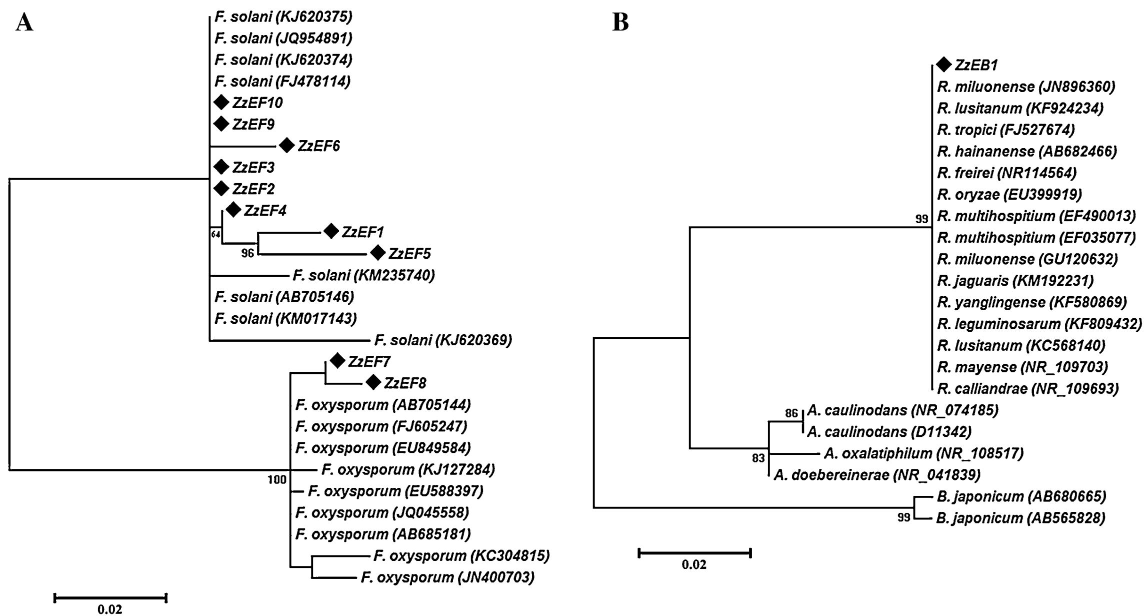

Fig. 2 Maximum likelihood tree based on ITS/ 16S rRNA gene sequences showing the relationship between *Z. zerumbet* **A** fungal (*Zz*EF1–*Zz*EF10) and **B** bacterial (*Zz*EB1) endophytes and other related microbial taxa. *Z. zerumbet* endophytes are indicated by prefixing with ◆ (*filled diamond*). The species origin with Genbank Accession Number of microbial sequences used for phylogenetic analysis are given at the end of each node. *Numbers* at nodes indicate bootstrap values (above 50 %) obtained from 1000 replications

Table 2 Antagonistic activity of endophytes isolated from *Z. zerumbet* rhizomes against *P. myriotylum* deteremined by dual culture assay

S. No.	Endophyte isolates	Percentage of inhibition (PoI; %)	Scale of antagonistic activity
1	ZzEF1	52.5 ± 1.25	3
2	ZzEF2	46.58 ± 2.67	2
3	ZzEF3	45.17 ± 0.29	2
4	ZzEF4	54.41 ± 3.81	3
5	ZzEF5	52.92 ± 1.17	3
6	ZzEF6	54.37 ± 2.65	3
7	ZzEF7	63.28 ± 2.53	4
8	ZzEF8	62.2 ± 2.58	4
9	ZzEF9	49.36 ± 3.40	2
10	ZzEF10	52.44 ± 2.41	3
11	ZzEB1	7.01 ± 3.31	1

Values are mean ± SE of three replications. The antagonistic activity was estimated on a 4-point scale based on PoI (%) values as: 1—Low antagonistic activity (<39); 2—Moderate antagonistic activity (40–49); 3—High antagonistic activity (50–59) and 4—Very high antagonistic activity (>60)

metabolite. Antagonistic activity exhibited by the isolated endophytes could be attributed to the production of bioactive metabolites as reported for endophytes isolated from other taxa (Castillo et al. 2002; Thongchai et al. 2003; Taechowisan et al. 2005). Even though *Fusarium* species possess the genetic potential to produce diverse secondary metabolites in aerial hyphae such as trichothecenes and napthaquinones (Greenhalgh et al. 1989), discontinuous distribution of biosynthetic genes account for differences in genetic potential of species to produce particular secondary metabolites (Ma et al. 2010, 2013). Production of these metabolites is also reported to be influenced by environmental factors and is strain and culture-condition dependent (Nesic et al. 2014). Wide ranges of biological activities are attributed to *Fusarium* derived trichothecenes and napthaquinones metabolites (Greenhalgh et al. 1989; Parisot et al. 1990; Brown and Proctor 2013; Nesic et al. 2014). However limited/no information is available till date on the antagonistic effect of *Fusarium* metabolite against economically significant oomycetous *P. myriotylum*. In this scenario, the identified isolates constitute promising resources for bioactive compounds with potent

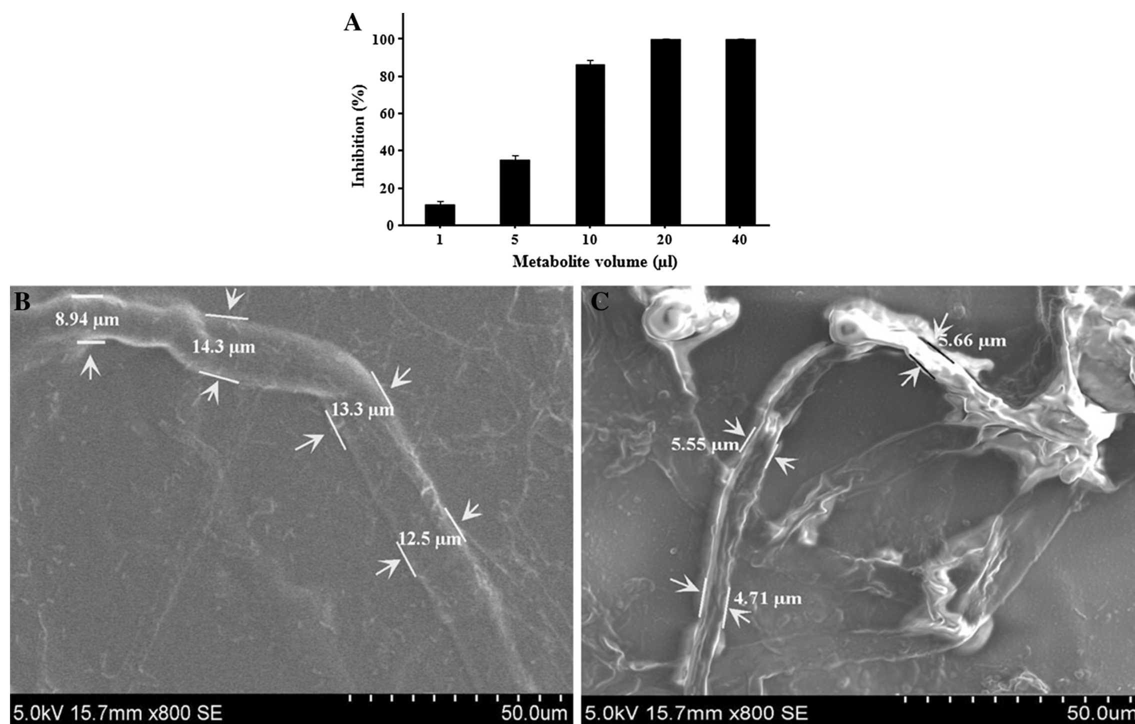

Fig. 3 Inhibitory effect of *Zz*EF8 metabolite extract on *P. myriotylum* hyphae. **A** Antagonistic effect determined by radial diffusion assays. Scanning electron micrographs (SEM) of *P. myriotylum* hyphae, **B** control hyphae and **C** following exposure to ZzEF8 metabolite extract for 2 h. Hyphal shrivelling (in µm) following exposure are indicated by *arrows*

anti-*Pythium* activity as evidenced in SEM analysis wherein hyphal deformities and collapse was observed following exposure to *Zz*EF8 metabolite. These morphological changes could be attributed to alterations in hyphal membrane permeability causing osmotic imbalances and leading to its antibiosis activity. Further investigations will be carried out in future to isolate and elucidate the active metabolite(s) for future development of sustainable alternatives to non-specific chemical fertilizers.

Conclusions

Endophytic fungi have gained significant importance as biocontrol agents for sustainable agricultural systems and are preferable over non-specific chemical fertilizers and pesticides due to low cost, effectiveness and environment friendly attributes. The identified endophytes obtained from the medicinally important and soft-rot resistant *Z. zerumbet* can thus be developed in future for control of *Pythium* spp. and constitute potentially important resource for exploring novel bioactive compounds.

Acknowledgments Authors are thankful to Director, NITC and Director, IISR for the research facilities provided. DK acknowledges MHRD (Ministry of Human Resource and Development, Govt. of India) for the research fellowship received.

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