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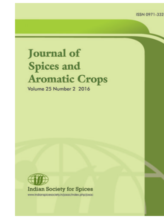

In vitro evaluation of fungal endophytes of black pepper against *Phytophthora capsici* and *Radopholus similis*

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Abstract

Experiment on *in vitro* screening of 125 endophytic fungi of black pepper against *Phytophthora capsici* indicated that 23 isolates showed more than 50% inhibition. The nematicidal activity of metabolites from endophytic fungi was also tested on *Radopholus similis* and the isolate BPEF73 (*Daldinia eschscholtzii*) showed highest mortality up to 60%. The isolates showing biocontrol potential were characterized based on internal transcribed spacer (ITS1 and ITS2) regions of rDNA. The sequence analysis of the isolates showed maximum identity with *Annulohypoxylon nitens* (BPEF25 and BPEF38), *Daldinia eschscholtzii* (BPEF41 and BPEF73), *Fusarium* spp. (BPEF72 and BPEF75), *Ceriporia lacerata* (BPEF81), *Diaporthe* sp. (BPEF11) and *Phomopsis* sp. (BPEF83). This is first report of endophytic association of above fungi in black pepper and the exploitation of their biocontrol potential against the major black pepper pathogens *P. capsici* and *R. similis.*

Keywords: biocontrol, black pepper, endophytic fungi, ITS rDNA sequence, *Phytophthora capsici*, *Radopholus similis*

Introduction

Black pepper (*Piper nigrum* L.) plants are affected by many diseases of which, the most destructive is the foot rot disease caused by *Phytophthora capsici* followed by plant parasitic nematodes and viruses (Anandaraj 2000). Many fungal and bacterial biocontrol agents are now available in market against *Phytophthora* foot rot but most of them are of rhizospheric origin and needs repeated soil applications and is obliged to compete with the native micro flora. Hence a plant based biocontrol alternative like endophytes have great significance. *Radopholus similis* the main causal organism of slow decline disease is another major threat to black pepper cultivation. Because of its migratory nature, control of *R. similis* is problematic; few bacterial biocontrol agents are available against *R. similis* of black pepper (Aravind *et al*. 2010) but fungal endophytes as biocontrol agents are unexplored. Endophytic fungi have recently been considered as an important resource for biocontrol agents (Sikora *et al*. 2008) against plant parasitic nematodes. The endophytic associations have been found in almost all plants studied (Schulz & Boyle 2005) and they may occupy the space available in leaves (Arnold 2007), stems and roots of the plants (Arnold *et al*. 2001).

Biocontrol potential of endophytes has been observed in many hosts. For example, endophytic fungi have been found to protect tomatoes (Hallman & Sikora 1996) and bananas (Sikora *et al*. 2008) from nematodes, beans, barley (Boyle *et al*. 2001), rice (Naik *et al*. 2009), fennel, lettuce, chicory and celery (D'Amico *et al*. 2008) against fungal pathogens and so on. Hanada *et al*. (2010), reported that endophytic fungal isolates were effective in suppression of the black-pod rot of cacao caused by *Phytophthora palmivora.* The mechanisms involved in biological control of endophytic fungi against plant pathogens include antibiosis (Morath *et al*. 2012; Kumar & Kaushik 2013), competition for nutrients and space (Narisawa *et al*. 2004), induction of defence response (Varma *et al*. 2012), and mycoparasitism (Gao *et al*. 2005). Black pepper endophytes remain largely unexplored with few exceptions (Aravind *et al*. 2010 & Sally *et al.* 2011). Considering these, a study was proposed to identify potential fungal endophytes against *P. capsici* and *R. similis.*

Materials and methods

Isolation of fungal endophytes from black pepper tissues

Healthy vines were selected from the black pepper varieties Panniyur1, Panniyur3, Sreekara, and Subhakara during 2009–2010. The stem, leaves and roots were collected from these vines and used for the isolation of endophytic fungi. Tissues were washed individually in running tap water and moved to the laminar air flow chamber where sections were cut with a sterile scalpel. These sections were surface-sterilized by dipping in 0.5% sodium hypochlorite for 2 min, then treated with 70% ethanol for 2 min and rinsed in sterile distilled water thrice followed by drying on sterile filter paper (Arnold *et al*. 2001). The edges of each tissue were cut off and discarded the remaining sterile tissues measuring around 2 × 3 mm were used for endophytic fungi isolation. To ensure surface sterilization, the sterilized tissue segments were pressed on to the surface of MEA medium and the final wash solution were pour plated on MEA and incubated for 30 days at room temperature under dark with periodic observations at every 24 h (Schulz *et al*. 1993). For isolation, the sterilized tissues were individually placed in petri dishes containing malt extract agar (MEA) amended with 0.1% stock antibiotic solution (0.02 g each tetracycline, streptomycin and penicillin in 10 mL sterile distilled water and filter sterilized). The plates were incubated as mentioned above.

In vitro *screening of endophytic fungal isolates against* P. capsici

Virulent *P. capsici* isolate no: 05-06 was obtained from National repository of *Phytophthora*, ICAR-Indian Institute of Spices Research, (ICAR-IISR) Kozhikode India. Mycelial disc of 5mm diameter cut from both endophytic fungi and *P. capsici* were placed 4 cm apart in petri dishes with equal distance from the periphery. Control plates with *P. capsici* alone were maintained and each treatment was replicated thrice. Percentage inhibition was calculated using the formula, $PI = (C–T) / C \times 100$ were PI = percentage inhibition; C = radial growth of the pathogen in control (mm); T = radial growth of the pathogen in dual culture (mm). Observations on the interactions were recorded from 72 h after inoculation. Three types of antagonistic activities were recorded: (1) Antibiosis: mycelial inhibition of *P. capsici* by endophytic fungi (2) competition for substrate: overgrowth of one organism by another; and (3) mycoparasitism: observation on direct parasitism of pathogen hyphae through microscope. Potential endophytic fungal isolate was selected from 125 isolates based on *in vitro* antagonistic activities.

Effect of volatile and non-volatile metabolites of endophytic fungal isolates on radial growth of P. capsici

The effects of volatile metabolites of the endophytic fungal isolates on mycelial growth of *P. capsici* were tested by using the method described by Dennis and Webster (1971). Two bottom portions of Petri plates containing potato dextrose agar (PDA) were inoculated with a 5 mm disc *P. capsici* and the test endophytes respectively and both PDA plates were placed facing each other and sealed with cellophane adhesive tape. Both Petri plates with pathogen served as control. The observations on the radial growth of the pathogen were recorded after 72 h of incubation. The colony diameter of the pathogen in the treatment in comparison with that of control gave percentage growth inhibition. For non volatile metabolite assay; culture filtrate of endophytic fungi grown on potato dextrose broth (PDB) at 28^{0}C with continuous shaking at 110 rpm were collected after 15 days and sterilized using 0.2 µm pore sized membrane filter. Sterilized filtrate was added to molten PDA at the rate of 1 mL/ 100 mL, and dispensed in to five 90 mm petri plates. Mycelial disc of *P. capsici* (5mm) was cut from the growing edges of 72 h old culture and inoculated at the centre. Plates without culture filtrates served as the control. The experiment was done in triplicate and was incubated for 72 h at room temperature.

Testing the nematicidal activity of metabolites of endophytic fungal isolates on R. similis *under* in vitro

The black pepper endophytic fungal isolates which showed more than 70% *in vitro* inhibition against *P. capsici* were grown in PDB for 15 days under dark at 28^{0}C with continuous shaking at 110 rpm. The crude metabolites, which diffuse in the medium, were collected using sterile muslin cloth and the mycelium was discarded and the extracts were filter sterilized using 0.2 µm pore sized membrane filter. 1 mL of extract was added to each well of microtiter plates and inoculated with 10 live *R. similis* (second stage juveniles) collected from nematode culture maintained on carrot culture. In control 1 mL filtered PDB was added instead of extract and a sterile water control was also kept. Three replications were maintained for each treatment. The plates were observed under a stereomicroscope for viable nematodes at 24 h intervals for 3 days. After 72 h incubation the plates were observed under a stereomicroscope by adding 20 µL of 1N NaOH (Aravind *et al*. 2010). The results were recorded and the percentage mortality of nematodes over the control was calculated.

Identification of the endophytic fungal isolates

PCR amplification of ITS region and sequencing

DNA of endophytic fungus was extracted following protocol of Cooke & Duncan (1997). The process involved grinding 500 mg of mycelium in 750 µL STE buffer (1 M Tris, 5 M NaCl, 0.5 M EDTA, 10% SDS) using micropestle. After centrifugation at 12000 rpm for 5 minutes, the aqueous phase was collected and extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Further extraction was repeated using chloroform: isoamyl alcohol (24:1). DNA was pelletted using isopropanol by centrifugation at 12000 rpm for 10 minutes. The pellet was washed in 70% ethanol and the dried pellet was resuspended in 50 µL of TE buffer. The quantity and quality of DNA was estimated by UV-spectrophotometer and by agarose gel electrophoresis. The polymerase chain reaction of the ITS region of the nine isolates were done using the primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'). The reactions were carried out in a total volume of 25 µL containing 50 ng of genomic DNA, 20 pmol of each primer, 10 mM of dNTPs, 1.5 unit of *Taq* polymerase in 1x PCR reaction buffer and 1.5 mM $MgCl_2$. Amplification was performed in a programmable thermal cycler (Eppendorf) with the following conditions: initial denaturation at 94^{0}C for 3 min, 30 cycles of denaturation at 94^{0}C for 30 sec, primer annealing at 55^{0}C for 30 sec, extension of annealed primer at 72^{0}C for 1 min and a final extension at 72^{0}C for 10 min. The amplicons were excised and were purified using Gen elute gel extraction kit (Genei, Bangalore). Purified PCR products were sequenced in the ABI DNA sequencer (Xcelris labs, Ahmadabad). Sequence similarity searches were performed using BLAST (Altschul *et al*. 1990) and submitted to NCBI (National Center for Biotechnology Information) GenBank and IISR data base. Accession numbers were assigned based on similarity in BLAST.

Pathogenicity testing of the endophytic fungal isolates

Pathogenicity of the isolates was tested by detached leaf assay using two month old susceptible black pepper variety Sreekara having 5-6 leaves. The second and third leaves (from the top) were kept with abaxial side facing up on moist chamber. Mycelial discs of 3 mm size from 7 days old endophytic fungal culture was inoculated on the leaf and incubated at room temperature for seven days, *P. capsici* inoculated leaves served as control and the experiment was replicated thrice. Observations were made at 24 h intervals after inoculation to monitor symptom developments.

Statistical analysis

Data were analyzed for significant differences by analysis of variance (ANOVA) with the statistical package SAS software (Version 9.3) and subjected to mean separation by the Least Significant Difference (LSD) test, P<0.05.

Results and discussion

Isolation of fungal endophytes from black pepper tissues

Isolations were made from plates that were negative for fungal growth in sterility checking plates. The incubation time ranged from 9-24 days. A total of 125 endophytic fungi (Table 1) were obtained. There were 59 isolates from stem, 38 from root and 28 from leaf (Fig. 1). Fungi growing out from the plant tissues were transferred to fresh MEA medium and reference name (BPEF) was assigned to each isolate. Whenever more than one type of mycelium was noticed, such cultures were processed only after purification. Cultures were preserved on PDA slants and stored at 15^{0}C in BOD incubator.

In vitro *screening of endophytic fungal isolates against* P. capsici

Dual plate assay

The maximum mycelial inhibition of 78% was recorded by three isolates BPEF81 (*Ceriporia lacerate*), BPEF83 (*Phomopsis* sp.) and BPEF11 (*Diaporthe* sp.) followed by 75% inhibition by BPEF73 (*Daldinia eschscholtzii)* (Table 2). BPEF25 (*Annulohypoxylon nitens*) and BPEF75 (*Fusarium* sp.) were equally effective against *P. capsici* (74%) (Fig. 2). Among the nine isolates four BPEF81, BPEF83, BPEF11and BPEF73 over grew *P. capsici* within 48 h and three isolates BPEF38 (*Annulohypoxylon nitens*), BPEF25 and BPEF41 (*Daldinia eschscholtzii)* over grew the pathogen after 72 h of incubation. Except BPEF72 (*Fusarium* sp.) and BPEF75 (*Fusarium* sp.), substrate competition was observed in all the isolates tested. Seven isolates were positive for antibiosis (Table 3) and four isolates showed mixed interactions (both antibiosis and competition). Mycoparasitism was observed in only one isolate, BPEF83 (*Phomopsis* sp.).

Effect of volatile and non-volatile metabolites of endophytic fungal isolates on radial growth of P. capsici

In the assay for volatile metabolites, after 72 h of incubation maximum growth inhibition was observed (Fig. 3) in the isolate BPEF38 (34.69%)

Table 1. Endophytic fungal isolates of black pepper varieties

State	Place of collection	Black pepper variety	Number of isolates obtained from			Total isolates
			Stem	Root	Leaf	
Kerala	Chelavoor, Kozhikode	Sreekara	8	8	2	18
Kerala	Chelavoor, Kozhikode	Panniyur 1	9	4	3	16
Kerala	Chelavoor, Kozhikode	Subhakara	8	5	6	19
Kerala	Peruvannamuzhi, Kozhikode	Panniyur 3	8	3	4	15
Kerala	Peruvannamuzhi, Kozhikode	Sreekara	10	7	4	21
Karnataka	Sakaleshpur, Hassan	Panniyur 1	8	4	5	17
Karnataka	Mudigere, Chikkamagaluru	Panniyur 1	8	7	4	19
Total isolates			59	38	28	125

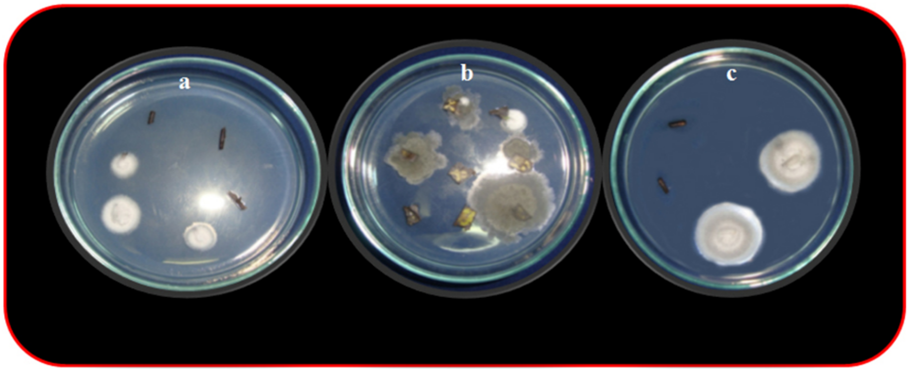

Fig. 1. Growth of endophytic fungi from (a) root (b) leaf and (c) stem of black pepper on MEA plates

Table 2. Biocontrol potential of endophytic fungal isolates against *P. capsici*

Endophytic fungal isolate	Source of tissue and variety (Identity/isolate number)	Mycelial inhibition(%) used for isolation
Diaporthe sp. (BPEF11)	Stem/Panniyur1	78.07(62.03) *
Annulohypoxylon nitens (BPEF25)	Stem/Panniyur1	74.14(59.34) *
Annulohypoxylon nitens (BPEF38)	Stem/Panniyur1	70.03(56.79) *
Daldinia eschscholtzii (BPEF41)	Stem/Panniyur1	72.00(58.05) *
Fusarium sp. (BPEF72)	Stem/Subhakara	70.00(56.79) *
Daldinia eschscholtzii (BPEF73)	Stem/Subhakara	75.00(60.00) *
Fusarium sp. (BPEF75)	Root/ Subhakara	74.15(59.34) *
Ceriporia lacerata (BPEF81)	Leaf/ Subhakara	78.88(62.58) *
Phomopsis sp. (BPEF83)	Leaf/ Subhakara	78.67(62.44) *
LSD ($P<0.05$)		0.9943

*Figures in the bracket are arc sine transformed

Table 3. *In vitro* screening of endophytic fungal isolates against *P. capsici*

Isolates	Biocontrol interaction of the isolates against *P. capsici* (after 72 h of incubation)		
	Competition	Antibiosis	Mycoparasitism
Diaporthe sp. (BPEF11)	+	+	-
Annulohypoxylon nitens (BPEF25)	+	+	-
Annulohypoxylon nitens (BPEF38)	+	+	-
Daldinia eschscholtzii (BPEF41)	+	+	-
Fusarium sp. (BPEF72)	-	+	-
Daldinia eschscholtzii (BPEF73)	+	-	-
Fusarium sp. (BPEF75)	-	+	-
Ceriporia lacerata (BPEF81)	+	+	-
Phomopsis sp. (BPEF83)	+	-	+

'+' and '-' signs indicate the presence and the absence of interaction respectively. In competition, '+' indicates the over growth of the pathogen by the isolates, in antibiosis, '+' indicates mycelial disintegration and zone of lysis and in mycoparasitism '+' indicates coiling of hyphaea of *P. capsici* by the endophytic fungal isolates.

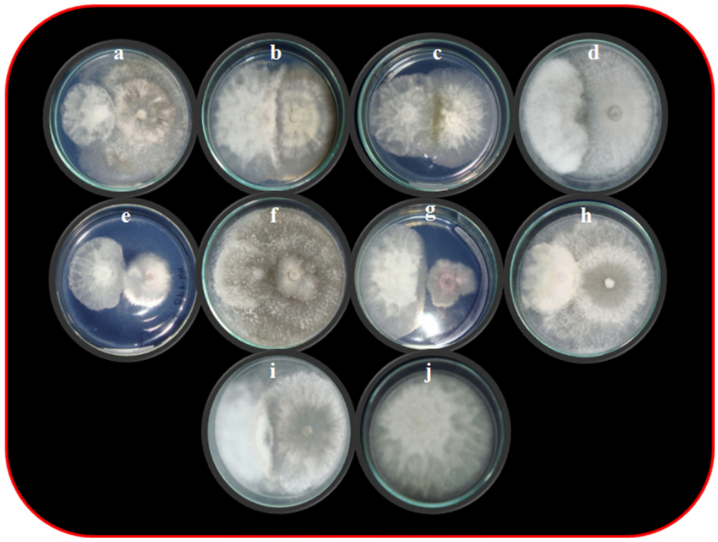

Fig. 2. Dual culture plates showing the inhibition of mycelia of *P. capsici* by endophytic isolates (a) BPEF11, (b) BPEF25, (c) BPEF38, (d) BPEF41 (e) BPEF72, (f) BPEF73, (g) BPEF75, (h) BPEF81, (i) BPEF83 and (j) *P. capsici* (Control).

followed by BPEF25 (31.07%). No growth inhibition was observed in BPEF4 and BPEF73. In the case of non-volatile metabolites production maximum growth inhibition was observed in BPEF25 (31.44%) followed by BPEF11 (24.94%). No effects were seen in BPEF73 and BPEF75.

Testing the nematicidal activity of metabolites of endophytic fungal isolates on R. similis *under* in vitro

Out of the nine isolates tested five isolates (BPEF11, BPEF73, BPEF75, BPEF81 and BPEF83) showed nematode mortality (ranging from 20 – 60%) after 72 h of incubation. The short listed five isolates were further assayed for mortality in 24 h intervals (Fig. 4). The maximum mortality was observed in BPEF73 (*Daldinia eschscholtzii*) followed by BPEF75 (*Fusarium* sp.). No mortality was observed in sterile distilled water as well as PDB controls even after 72 h of incubation (Fig. 5).

Identification of the endophytic fungal isolates

The two sporulating isolates were identified as *Fusarium* sp. and further confirmed by ITS

Table 4. Identification of the endophytic fungal isolates

Isolate	Identification based on BLAST search of ITS sequences	Gene Bank accession number	Identity match(%)
BPEF11	*Diaporthe* sp.	KF219919	98
BPEF25	*Annulohypoxylon nitens*	KF151846	99
BPEF38	*Annulohypoxylon nitens*	KF254768	99
BPEF41	*Daldinia eschscholtzii*	KF151848	99
BPEF72	*Fusarium* sp.	KF151847	99
BPEF73	*Daldinia eschscholtzii*	KF151849	99
BPEF75	*Fusarium* sp.	KF151850	98
BPEF81	*Ceriporia lacerata*	KF151851	98
BPEF83	*Phomopsis* sp.	KF219920	99

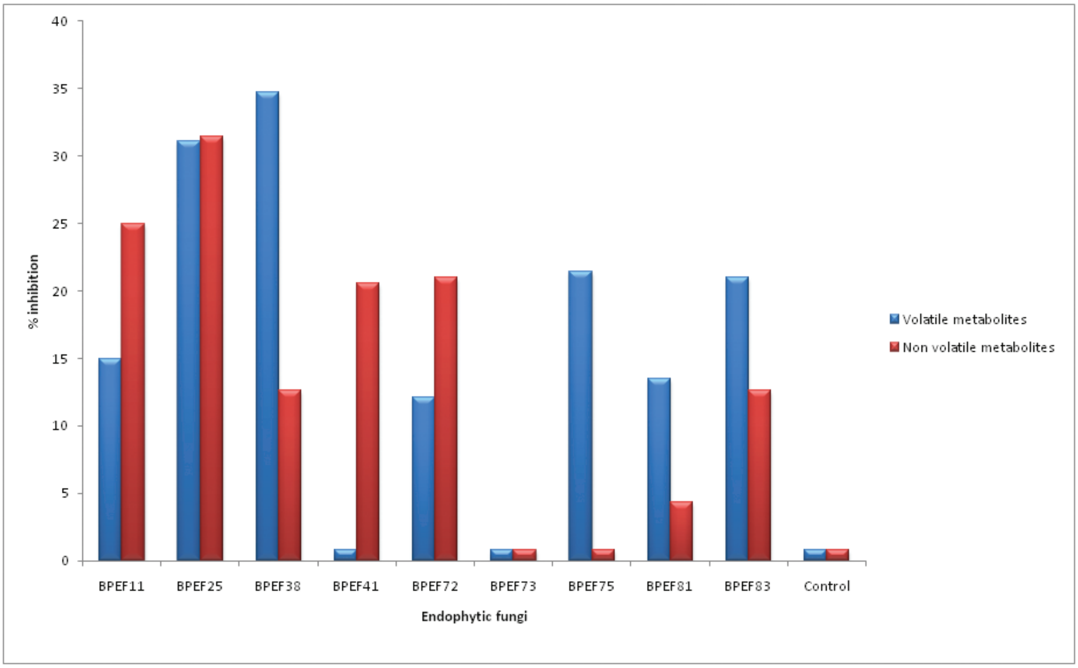

Fig. 3. Effect of volatile and non-volatile metabolite of endophytic fungi on growth of *P. capsici.* Sine Arc transformed values for each replication was used, ANOVA and DMRT (LSD at P ≤0.05 for volatile metabolite = 1.2 and for non volatile metabolites = 1.269) were performed.

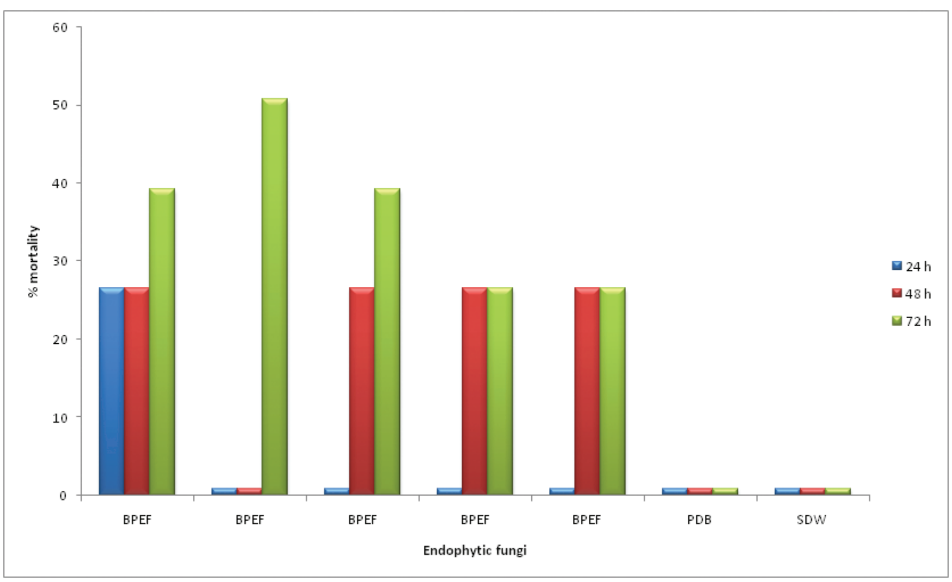

Fig. 4. Percentage mortality of *R. similis* incubated with metabolites (at 24 h intervals)

sequencing. All the non sporulating isolates were identified based on ITS sequencing. An amplicon of 600 bp was obtained for all the isolates. Blast search of the isolates showed similarity with other reported endophytic fungi (Table 4). The sequences were deposited in NCBI. The accession numbers are given in Table 4. Name was assigned based on BLAST search results of ITS sequences of most similar sequences and other reported endophytes.

Pathogenicity of the isolates

None of the isolate caused any symptom when incubated even up to seven days whereas

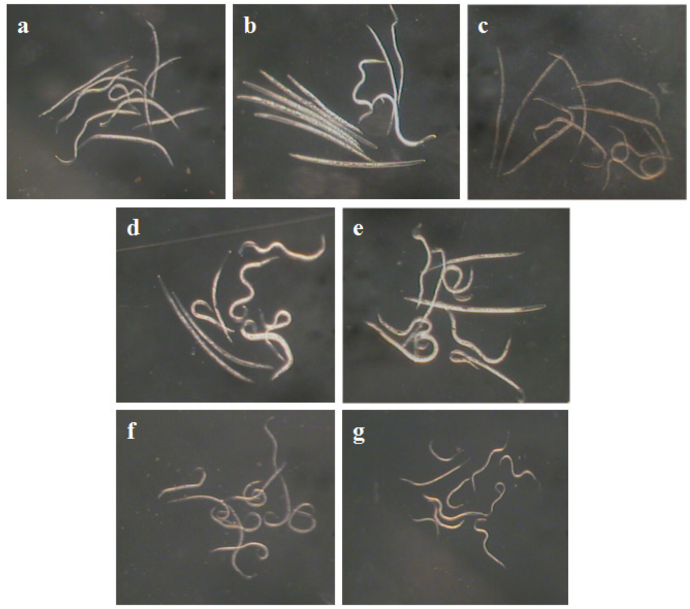

Fig. 5. Bioassay of culture filtrate against of *R. similis* after 72h of incubation. Here the dead nematodes assume straight posture (a) BPEF11 (b) BPEF73 (c) BPEF75 (d) BPEF81 (e) BPEF83 (f) SDW and (g) PDB.

P. capsici could cause symptoms within 24 h and the leaf got fully infected by 7 days.

This study indicated that black pepper harbours endophytic fungal flora belonging to the genera *Annulohypoxylon*, *Daldinia*, *Fusarium*, *Ceriporia*, *Diaporthe* and *Phomopsis* which supports the fact that no vascular plant deprived of endophytic fungi could be seen and endophytes are widespread in all major taxonomic groups of plants living under various environments (Carroll 1988; Clay 1993; Tondje *et al*. 2006; Arnold 2007; Rodriguez *et al.* 2009). Endophytic fungi have recently been considered an important resource for screening biocontrol agents to suppress plant pathogens (Sikora *et al*. 2008; Naik *et al*. 2009; Hanada *et al.* 2010). Endophytic fungi isolated from black pepper have been tested for their antagonistic potential against two major pathogens of black pepper *P. capsici* and *R. similis*. The isolates which showed more than 70% *In vitro* mycelial inhibition against *P. capsici* were studied for major biocontrol traits of endophytes like competition, antibiosis and mycoparasitism (Narisawa *et al*. 2004; Bailey *et al*. 2008; Morath *et al*. 2012; Kumar & Kaushik 2013). The isolates *Ceriporia lacerata* (BPEF81), *Phomopsis* sp. (BPEF83) and *Diaporthe* sp. (BPEF11) are equally effective against *P. capsici in vitro*. The most common anti-oomycete mechanism observed in this study was substrate competition followed by antibiosis. Limited inhibition of *P. capsici* was observed by volatile and non-volatile metabolites of endophytes (less than 40%). The nematicidal activity of metabolites from endophytic fungi was also tested on *R. similis* and the isolate BPEF73 (*Daldinia eschscholtzii*) showed highest mortality (60%). Nel *et al.* (2006), studied the potential of non-pathogenic *Fusarium* spp. for the biological control of plant diseases.

Traditionally fungal identification was done based on morphological characteristics but most of the endophytic isolates do not sporulate in cultures (Petrini 1991; Guo *et al*. 2000; Photita *et al*. 2001; Cannon and Simmons, 2002). There were reported techniques for inducing sporulation in fungi (Guo *et al*. 2000) but they were time-consuming to make a complete identification. For identification of fungi molecular tool like ITS rDNA sequencing was reported useful (White *et al*. 1990). In this study ITS rDNA sequencing was used for taxonomic identification of non sporulating endophytic isolates. Among the nine isolates of endophytes from black pepper that were showing inhibitory effect on two major pathogens of black pepper namely *P. capsici* and *R. similis* except *Fusarium*, seven of them were nonsporulating types, based on ITS rDNA sequencing they were identified. Since any of the efficient endophytic isolates showed any pathogenic reaction on black pepper on artificial inoculation there is a great potential to use them as biocontrol agents for the management of *P. capsici* and *R. similis* infections in black pepper.

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