Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Fusarium solani* isolated from bark of Himalayan yew

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An endophytic fungus isolated from *Taxus baccata* bark displayed considerable antimicrobial activity. The fungus was identified as *Fusarium solani* based on morphological and molecular characterization. The metabolite showed activity against both bacterial and fungal pathogens. The optimum conditions for metabolite production were found to be 30±1°C, slightly acidic pH and incubation period of 10 days. The crude metabolite was separated into two fractions by column chromatography over silica gel (60–120 mesh) using a mixture of petroleum ether and ethyl acetate (20:1) as the eluent. The metabolite was characterized and identified by Gas-Chromatography Mass-Spectrophotometry (GC-MS) analysis due to its volatile nature. The main components were 1-tetradecene, 8-octadecanone, 8-pentadecanone, octylcyclohexane and 10-nonadecanone. Phylogenetic analysis of the fungus with other endophytic fungi producing bioactive metabolites revealed its close affinity with an *F. solani* isolate that produced taxol. The metabolite produced by the endophytic fungus could be an alternative source of antimicrobial agents against clinical pathogens.

Keywords: Antimicrobial metabolite – *Fusarium solani* – Phylogenetic analysis – *Taxus baccata*

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Introduction

Increasing levels of antibiotic resistance in both pathogenic and nonpathogenic bacteria has spurred the search for new antibiotics to control diseases. Realizing the capability of microorganisms to produce diverse bioactive molecules and the existence of unexplored microbial diversity, research is underway to isolate and screen microbes from diverse habitats and unique environments for discovery of novel metabolites. In recent years, the search for new metabolites has been directed towards microbes residing inside plants tissues called endophytes. By definition, endophytes are those microbes colonizing healthy tissues of plants, at least for a part of their life cycle, without causing apparent disease symptoms in their host (Petrini 1991). The ubiquity of endophytes in the plant kingdom is well established; they have been isolated from all species investigated so far (Arnold et al. 2001). Analysis of any plant material may result in the discovery of a range of different endophytic fungi. Many of these fungi might appear to be specific to a particular host. In addition, environmental and edaphic conditions are expected to affect the nature and the population of endophytes.

Work carried out so far regarding the role of endophytes in host plants indicate that they

can stimulate plants growth, increase disease resistance, improve the plants ability to withstand environmental stresses and recycle nutrients (Sturz et al. 2000, Strobel 2002). Endophytes that reside in leaves and stems of plants contribute to the successful survival of the host. The array of alkaloids and other chemicals synthesized by the endophytes endow the plant with more resistance to nematodes, insect herbivores and livestock (Schardl et al. 2004). In addition, endophytes are also recognized as rich sources of secondary metabolites of multifold importance (Tan & Zou 2001, Strobel & Daisy 2003). There is a need to investigate fungal endophytes from medicinal plants because it has been hypothesized that these plants harbour some distinct and rare microbes that mimic the chemistry of their respective hosts and synthesize identical bioactive natural products or derivatives that are more bioactive than the one produced by the host. Many workers have demonstrated that the endophytes isolated from medicinal plants are excellent producers of strong fungicidal, bactericidal and cytotoxic metabolites (Radu & Kqueen 2002, Wang et al. 2007).

Amongst various high value medicinal plants of the Himalayan region, *Taxus baccata* ssp. *wallichiana* (the only *Taxus* species in India and commonly known as Himalayan yew) has gained considerable importance as a source of the anti-cancer drug taxol (paclitaxel), a diterpenoid, which was first isolated from the stem bark of *T. brevifolia* (Purohit et al. 2001). Besides, many fungal endophytes associated with *Taxus* spp. have been demonstrated to produce important bioactive metabolites (Strobel et al. 1997, Wang et al. 2000). A similar study conducted on fungal endophytes associated with *T. baccata* from this part of the world result in isolation of an endophytic fungus with considerable antimicrobial activity against some clinically significant microorganisms. In this study we identified the fungus by molecular analysis in addition to morphological characterization. We also optimized, characterized and identified the antimicrobial metabolite by Gas-Chromatography Mass-Spectrophotometry (GC-MS). An attempt was also made to investigate the phylogenetic relationships of the fungus to other fungal endophytes producing bioactive metabolites using nucleotide sequences of rDNA ITS region.

Materials and methods

Isolation and identification of the fungus

The fungus used in this study was obtained as an endophyte from inner bark of *Taxus baccata* collected from Dibang Valley of Arunachal Pradesh, a part of Eastern Himalaya, India. The procedure used to isolate this organism has been described previously and included surface sterilization technique by immersing the bark sequentially in 70% ethanol for 3 min and 0.5% sodium hypochloride (NaOCl) for 1 min and rinsed thoroughly with sterile distilled water, carefully excising the inner tissues and placed on petri plates containing potato dextrose agar (PDA) and water agar (WA) media (Tayung & Jha 2006). The fungus was induced to sporulate by inoculating in malt extract agar medium and identified both morphologically and based on ITS rDNA sequence analysis. It was among several endophytic isolates that displayed antimicrobial activity against some clinically significant microorganisms and therefore, was selected for further study.

Cultivation and metabolite extraction

The fungus was cultivated on potato dextrose broth by placing agar blocks of pure culture (3 mm in diameter) of actively growing culture in 500 ml Erlenmeyer flask containing 200 ml of the medium. The flask was incubated in BOD shaking incubator for 15 days at 24±2°C with periodic shaking at 150 rpm. The fermentation broth of the endophyte was filtered through cheesecloth to remove the mycelial mats. The filtrate was extracted thrice with ethyl acetate at room temperature. The pooled extract after drying over anhydrous $MgSO_4$, was evaporated in a rotary vacuum evaporator. The crude extract was then dissolved in dimethyl sulphoxide (DMSO) for antimicrobial bioassay.

Determination of antimicrobial activity

The tested pathogens include three gram positive bacteria namely *Bacillus subtilis, Staphylococcus epidermidis* and *S. aureus,* three gram negative bacteria namely *Klebsiella*

pneumoniae, Shigella flexneri and *Escherichia coli* and two pathogenic fungi *Candida albicans* and *C. tropicalis*. The antimicrobial activity of the metabolite was determined by agar cup diffusion method with each agar cup (6 mm diameter) filled with 100 µL of the crude extract that was dissolved in DMSO. The control cup was filled with only DMSO. The plates were incubated at 35±1°C for 24 h and the zone of inhibition was measured and compared with the control. Three replicates were maintained in each case. The magnitude of antimicrobial action was assessed by the diameter (mm) of inhibition zones and compared with co-assayed antibiotics tetracycline and fluconazole were used as antibacterial and antifungal agents.

Process optimization for the production of active metabolite

Different process parameters like biomass, effect of temperature, pH and incubation periods were studied for optimum production of bioactive metabolite. The activity of the liquid culture was tested against *Bacillus subtilis* and *Klebsiella pneumoniae* by agar cup diffusion method. Biomass accumulation was determined by drying the mycelial mat at 70°C until a constant weight was obtained and expressed as mg/ml. Temperatures from 10–45°C (at difference of 5°C), incubation periods from 2–15 days (at difference of 2 days) and pH ranging from 2–10 (at difference of one) were studied by inoculating the fungus in potato dextrose broth and the effect on metabolite production was observed after 2 weeks.

Isolation of genomic DNA, PCR amplification and sequencing

Total genomic DNA was extracted from mycelia of the fungus grown on PDA medium by using CTAB method (Cai et al. 2006) DNA amplification was performed by PCR. The PCR was set up using the following components: 5µL Buffer (10×), 3µl $MgCl_2$ (25mM), 1µL dNTPs (10mM), 1.5µL Taq Polymerase (5U), 1.5µL Forward primer (10µM), 1.5µL Reverse Primer (10µM), 3µL DNA template and 34.7µL distilled water. Initial denaturation was at 95°C for 5 min denaturation, annealing and elongation were done at 95°C for 1min, 52°C for 30 sec and 72°C for 1 min, respectively, in 45 cycles. Final extension was at 72°C for 10 min and hold at 4°C. For amplification of ITS-rDNA region ITS4 and ITS5 primers were used according to the method described by White et al. (1990). The PCR product, spanning approximately 500 – 600bp was checked on 1% agarose electrophoresis gel. It was then purified using quick spin column and buffers (washing buffer and elution buffer) according to the manufacturer's protocol (QIA quick gel extraction kit Cat No. 28706). DNA sequencing was performed using the above mentioned primers in an Applied Biosystem 3130xl analyzer.

Phylogenetic analysis

The sequence obtained was annotated using sequin software and submitted to NCBI GenBank database. It was aligned by Clustal W multiple sequence alignment program to other ITS rDNA sequences of endophytic fungal taxa obtained from GenBank database. All characters were equally weighted and unordered. Alignment gaps were treated as missing data. Phylogenetic analysis using MEGA4 was used to generate phylogenetic tree (Tamura et al. 2007). The optimal tree with the sum of branch length was 1.61623394. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 292 positions in the final dataset.

Fractionation and separation of the active metabolite

The crude metabolite was separated by TLC and purified into two fractions by column chromatography over silica gel (60–120 mesh) using a mixture of petroleum ether and ethyl acetate (20:1) as the eluent. The fractions were characterized and identified by GC-MS analysis due to volatile nature of the compound. Chromatography was performed on a DB-Wax capillary column (30 m × 0.25 mm ID and 0.25 µm film thickness). The electron

impact technique (70 eV) was used. The carrier gas was helium at a flow rate of 1.0 mL/min, and 1 μL of sample was injected. The injector and detector temperatures were 230°C and 200°C, respectively. The column oven was programmed as follows: initial temperature 60°C; initial time 2.0 min; program rate 10°C /min; final temperature 250°C; final time 10 min. The sample was dissolved in CH_2Cl_2 and a split injection technique was used. The identification of the compounds was based on comparison of their retention indexes (RI), obtained using n-alkanes (C_{11}–C_{31}), and retention time. They were also confirmed by comparison of their mass spectra with the NIST/NBS-Wiley library spectra and literature data. Relative percentage amounts were calculated from TIC by the computer.

Results

Isolation and identification of endophytic fungus

The fungus, isolated as endophyte from healthy inner bark of *Taxus baccata* grew as a white colony, reverse colorless, cottony and turning into pale white with violet margins on PDA medium. It produced septate, hyaline hyphae, simple or branched and numerous single-celled and clavate microconidia. The macroconidia were sickled-shaped, septate and less frequent. The morphological characters of the fungus revealed *Fusarium* sp., confirmed by molecular analysis. The rDNA of ITS region was amplified and the PCR product was bidirectionally sequenced using forward (ITS4) and reverse (ITS5) primers. Based on BLAST search analysis the fungus was identified as *Fusarium solani* (Mart.) Sacc. A living culture of the isolate is deposited in Microbial Type Culture Collection (MTCC) and Gene bank, Institute of Microbial Technology (IMTECH) Chandigarh, India (accession no. 9622). The sequence has also been deposited in GenBank of NCBI (accession no. FJ719812).

Antimicrobial activity of the crude metabolite

The crude metabolite was extracted from fermentation broth of the fungus by solvent extraction procedure. The metabolite exhibited strong to moderate antimicrobial activity against all the test pathogens (Table 1). To assess the magnitude of antimicrobial action, the metabolites were co-assayed with two reference antibiotics i.e., tetracycline as antibacterial and fluconazole as antifungal agent. The metabolite showed highest zone of inhibition against *Klebsiella pneumoniae* (27 mm) followed by *Shigella flexneri* (24 mm) whose antibacterial activity was almost similar to that of the positive control, tetracycline (Fig. 1). Among bacterial pathogens, the metabolite showed lowest activity against *E. coli* (16 mm) but the activity was greater than that of the co-assayed antibiotics. Further, the metabolite exhibited strong antifungal activity against *Candida albicans* (20 mm) but low activity against *C. tropicalis* (10 mm). Both these pathogens were resistant to the reference antifungal agent, fluconazole (Fig. 2).

Optimization process for metabolite production

The fungus was cultured aerobically at stationary condition in potato dextrose broth and effect of temperature, pH and incubation period were observed on metabolite and biomass production. Maximum growth (3.4 mg/mL) and optimum activity of the metabolite was recorded at incubation temperature of 30°C (±1). Biomass production and activity of the metabolite began at 15°C, reached a peak and then dropped considerably at 40°C (Fig. 3). A similar trend was observed for pH on growth and metabolite activity. Maximum growth (3.5 mg/ml) and optimum activity was recorded under slightly acidic conditions (Fig. 4). Incubation of 10–12 days was optimum for metabolite activity and 1011 days for maximum biomass production (3.9 mg/ml). Metabolite production started at 3 days, remained static after 10 days of incubation and then gradually declined (Fig. 5).

Characterization of the active metabolite

The crude metabolite (0.543 g) was separated into major and minor fractions by column chromatography over silica gel (60–120 mesh) using a mixture of petroleum ether and ethyl acetate (20:1) as the eluent. The major fraction 1 (F1) was a colorless liquid that weighed 0.166 g and minor fraction 2 (F2) was red and semi-solid mass that weighed 0.181 g.

Table 1 Antimicrobial activity of endophytic *Fusarium solani* isolated from *Taxus baccata*.

Test pathogen	Zone of inhibition (mm) (1mg ml^{-1})	Tetracycline (30mcg $disc^{-1}$)	Fluconazole (10mcg $disc^{-1}$)
Staphylococcus aureus	18.6±1.1	22.0±0	—
Staphylococcus epidermidis	20.3±0.5	24.0±0.5	—
Bacillus subtilis	18.3±1.5	24.3±0.5	—
Klebsiella pneumonia	27.0±1.7	30.0±0	—
Escherichia coli	16.3±0.5	8.6±1.1	—
Shigella flexneri	24.3±1.1	25.0±0	—
Candida albicans	20.3±0.5	—	—
Candida tropicalis	10.6±1.1	—	—

± SD – standard deviation; – No inhibition. Negative control: dimethly sulphoxide (the medium to dissolve the crude metabolites) 100µL/well; co-assayed antibiotics: tetracyline (30mcg/disc) for bacteria; fluconazole (10mcg/disc) for fungi.

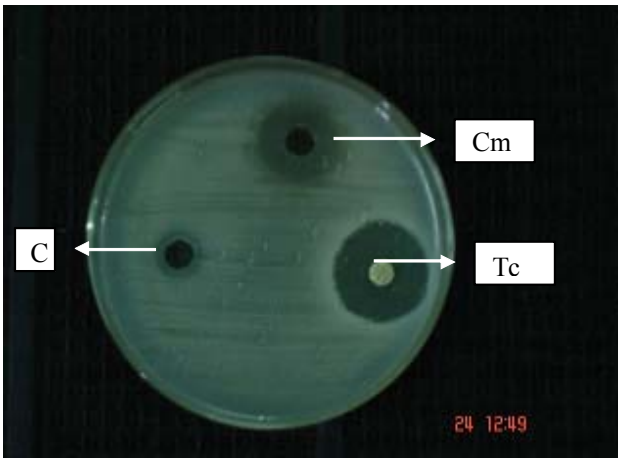

Fig. 1 – Antibacterial activity of the crude metabolites (Cm) against *Staphylococcus epidermidis*. Co assayed antibiotic tetracycline (Tc) and control (C).

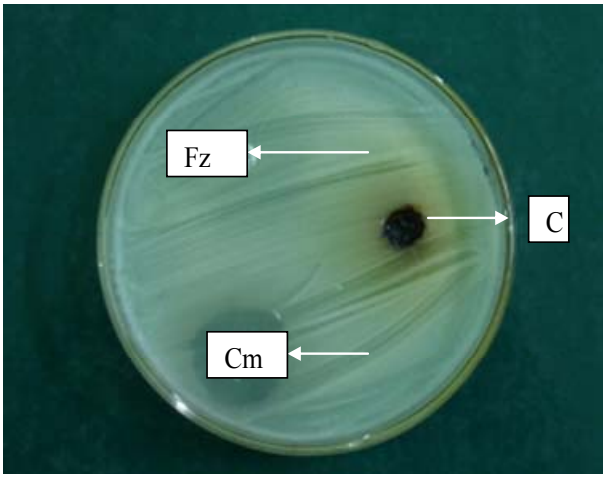

Fig. 2 – Antifungal activity of the crude metabolites (Cm) against *Candida albicans*. Co-assayed antibiotic fluconazole (Fz) and control (C).

Both fractions F1 and F2 were subjected to GC-MS analysis. The chromatogram showed that F1 was a mixture of at least 15 compounds (Fig. 6a). However, there were only three major components which accounted for 80% of the total mass. From mass spectral analysis five compounds were identified and they together accounted for at least 82% of the total mass. Composition of the remaining 16% could not be ascertained due to their low abundance. Similarly, GC-MS analysis of the F2 showed five compounds and of these only three major components, which together accounted for 84% of the total mass (Fig. 6b). The major identified compounds of F1 and F2 are presented in Table 2.

Table 2 Major identified compounds of fraction 1 and 2 produced by *Fusarium solani*

Compounds	RT (min)	Abundance (%)
Fraction-1		
Dodecene	4.19	6.5
Hexylcyclohexane	5.28	5.4
1-Tetradecene	10.77	43.8
Tetradecane	11.12	9.8
Octylcyclohexane	14.20	16.5
Fraction-2		
10-nonadecanone	8.76	12.95
8-Pentadecanone	2.96	29.68
8-Octadecanone	4.84	41.36

Phylogenetic analysis

The rDNA genes (partial 18S, ITS1, 5.8S, ITS2 and partial 28S) sequence of the *Taxus* isolate was compared to nineteen corresponding sequences of endophytic fungal taxa producing bioactive metabolites from different hosts and locations obtained from GenBank

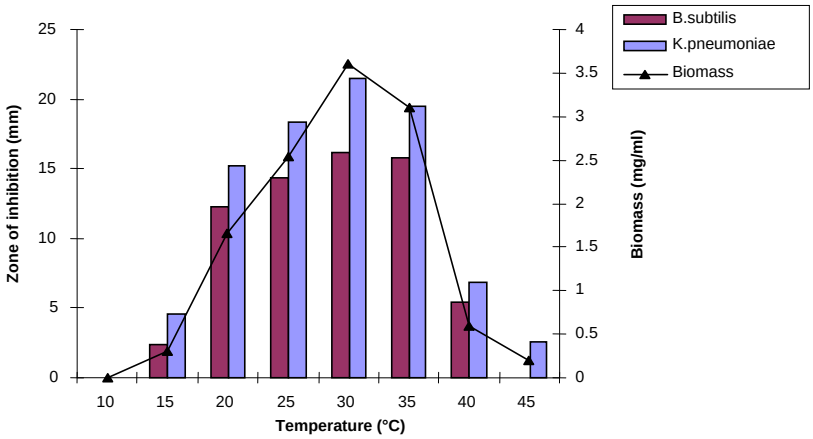

Fig. 3 – Effect of temperature (°C) on production of active metabolites by *Fusarium solani* DEF3

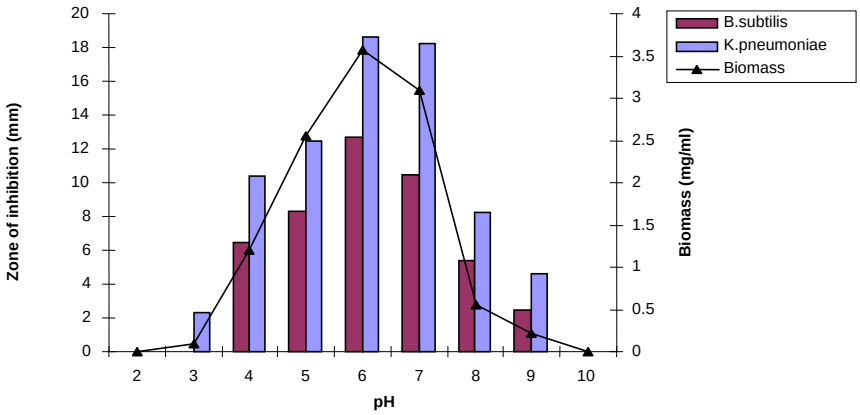

Fig. 4 – Effect pH on production of active metabolites by *Fusarium solani* DEF 3

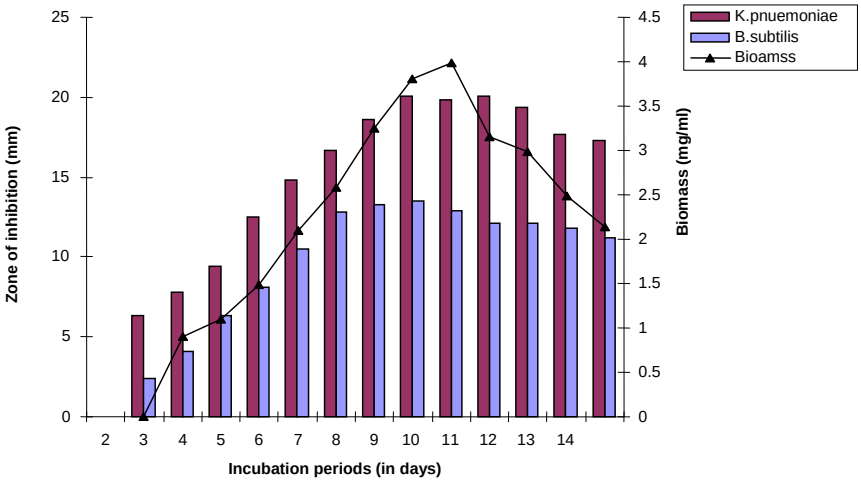

Fig. 5 – Effect of incubation period on production of active metabolites by *Fusarium solani* DEF3

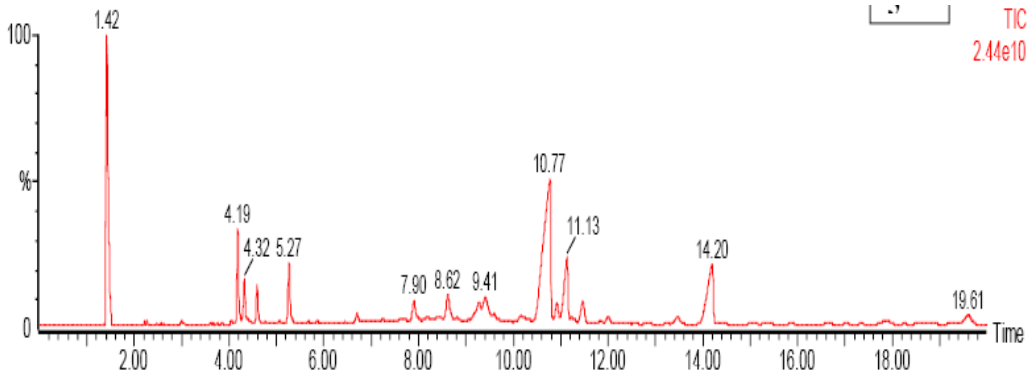

Fig. 6 (a)

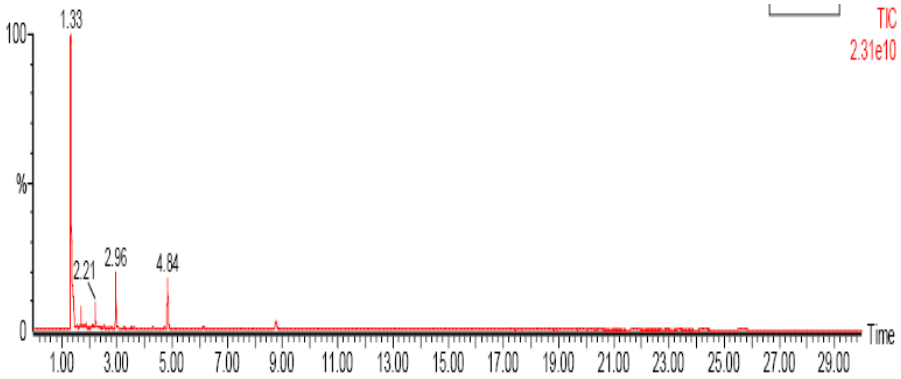

Fig. 6 (b)

Fig. 6 – GC-MS analysis of the metabolite **(a)** fraction-1 and **(b)** fraction-2

database (Table 3). A phylogenetic tree was generated by UPGMA method which showed four clades and one independent lineage (Fig. 7). The cladogram showed all endophytic fungi form distinct groups irrespective of their metabolite production. A major clade of all the *Fusarium* spp. is divided into two subclades. Subclade I and II forms clusters of *F. oxysporum* and *F. solani* respecttively producing anticancer and antimicrobial metabolites. Interestingly, both (FJ719812.1) and (EU442277.1) were obtained as endophytes from *Taxus* species. The latter was isolated from *T. chinensis*, China and was reported to produce taxol, an anticancer metabolite whereas *F. solani* (FJ719812.1) isolated from *T. baccata*, India was found to produce volatile metabolites with antimicrobial property.

Discussion

Many new and interesting bioactive metabolites such as antibiotics, antiviral, anticancer and antioxidant compounds, which are of pharmaceutical, industrial and agricultural importance have been reported and characterized from fungal endophytes. Strobel & Daisy (2003) suggested studying endophytic fungi since such plants may harbour unique and rare endophytes capable of producing important bioactive metabolites with multiple applications. *Taxus*, a gymnosperm is an important anticancer plant. Several endophytic fungi isolated from *Taxus* spp., worldwide have been reported to produce important bioactive metabolites (Wang et al. 2007). The endophyte from India was identified as *F. solani* both by morphological and molecular characterization based on rDNA gene sequences. Identification of *Fusarium* spp., at the subgenus level by morphological traits is difficult because of unstable mycelial pigmentation, shape and size of conidia feature all highly dependent on composition of the media and environmental conditions. Molecular techniques based on rDNA genes have been successfully applied to identify fungi particularly variable species like *Fusarium* (Cai et al. 2005). Most *Fusarium* spp. are soil-borne and widely distributed in nature as saprophytes and pathogens. Some species cause plant diseases with important economic

Table 3 List of endophytic fungal taxa producing bioactive metabolites and GenBank accession numbers of ITS sequences used in this study.

Species	Host	Metabolite	Biological activity	Country	GenBank Accession
Phoma medicaginis	__	Brefeldin	Antimicrobial	Germany	AY504634
Exserohilum rostratum	*Stemona* sp.	Hydroxymo-nocerin	Antiplamodial	Thailand	EU571210
Alternaria alternata	Seaweed	__	Antimicrobial	China	FJ618522
Alternaria sp.	*Polygonum senegalense*	__	Cytotoxic	Germany	EU143251
Phomopsis sp.	__	Ascosteroid A	Anticandida	Germany	DQ872669
Phomopsis sp.	*Azadirachta indica*	Lactones	__	China	EU256482
Fusarium solani	*Taxus baccata*	Mixed volatile compounds	Antimicrobial	India	FJ719812**
Fusarium solani	*Taxus chinensis*	Taxol	Anticancer	China	EU442277.1
Fusarium solani	*Taxus mairei*	Taxol	Anticancer	Taiwan	AY43380
Fusarium solani	*Apodytes dimidiate*	Campothecin	Anticancer	India	GQ465774
Fusarium sp.	*Discorea zingerberensis*	__	Antimicrobial	China	DQ446211
Fusarium sp.	*Thunbergia laurifolia*	__	Acanthoamoe-abicidal	Thailand	EU352873
Fusarium oxysporum	*Taxus cuspidate*	Taxol	Anticancer	S. Korea	AY555719
Fusarium oxysporum	*Nothapodytes nimmoniana*	Camptothecin,	Anticancer	India	FJ158124
Fusarium oxysporum	*Annona squamosa*	Polyketide	Anticancer/ Antimicrobial	China	EF488410
Fusarium oxysporum	*Acianthera teres*	__	Antimicrobial	Brazil	FJ605244
Fusarium oxysporum	*Dracaena cambodiana*	__	Antimicrobial	China	FJ449900
Fusarium oxysporum	*Discorea. zingerberensis*	__	Antimicrobial	China	DQ459007
Xylaria sp.	*Siparuna* sp.	Lactones	Antiplasmodial	Panama	EU016102
Penicillium glabrum	Seaweed	__	Antimicrobial	China	FJ618520
Colletotrichum sp.	__	Colutellin A	Immunosup-pressive	USA	EU330193

—no data available
**Fungus used in the present study

impacts while others cause severe human infections (Guarro & Gene 1995). *Fusarium* spp. have been reported as endophytes from several plants with diverse biological activity (Shiono et al. 2007, Kour et al 2008, Deng et al. 2009). This suggests their ubiquity as endophytes within the plant kingdom and provides an opportunity to discover novel bioactive metabolites.

Fusarium solani has been reported as an endophyte from several yew species with production of the anticancer metabolite taxol in vitro (Chakravarthi et al. 2008, Li et al. 2005). With the discovery of taxol producing endophytic fungus, *Taxomyces andraenae* from pacific yew bark, fungal endophytes from yew are being extensively investigated for taxol production. However, there is little information on antimicrobial metabolites from these endophytes. The antimicrobial activities of *F. solani* isolated as endophyte from *T. baccata* bark suggests that yew species may harbour endophytes that could produce antimicrobial metabolite other than taxol. The activity of the metabolite was found to be similar to that of reference antibacterial agent, tetracycline and also exhibited antifungal activity against fungal pathogens resistant to reference antifungal agent, fluconazole. This suggests the metabolites are broad spectrum in nature and that they have potential application as antimicrobial agents. The optimum temperature for metabolite production was found to be 30±1°C. In many instances extraction of active metabolite from endophytic fungi were done at 28±1°C (Wang et al. 2007). This indicated that

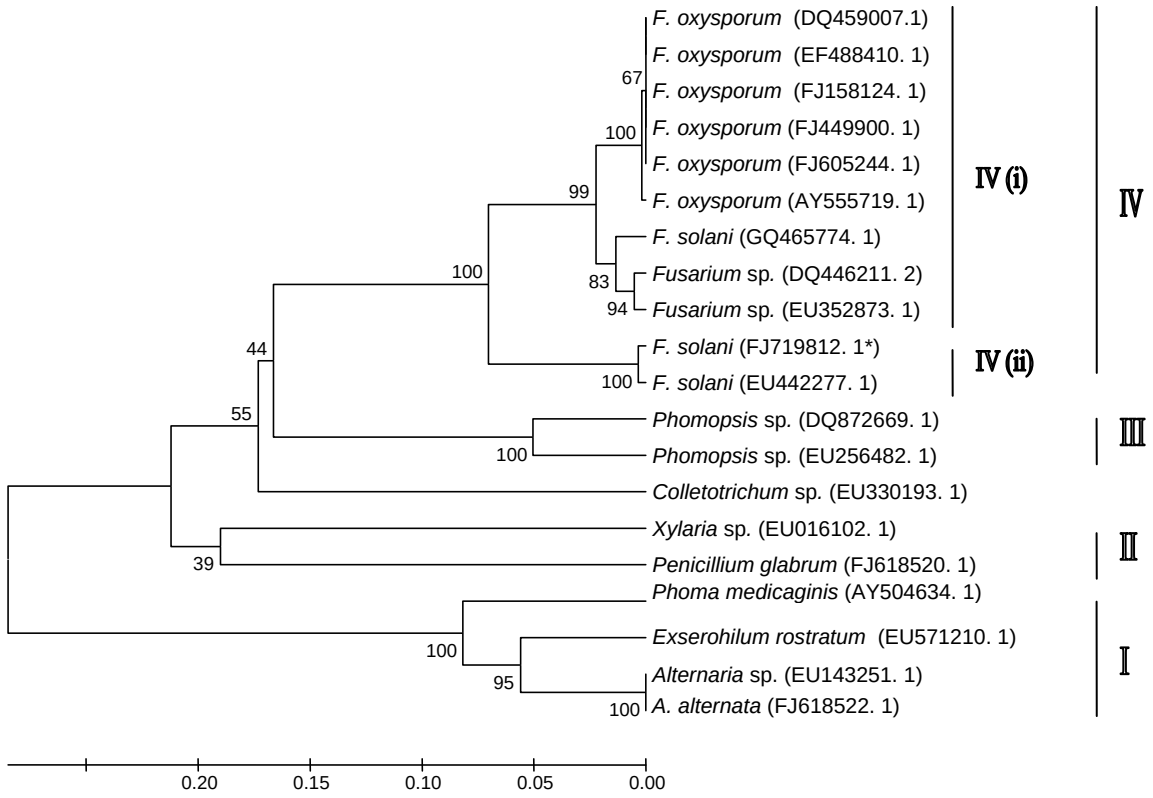

Fig. 7 – Phylogenetic tree showing evolutionary relationships of 20 fungal taxa producing bioactive metabolites. The evolutionary history was inferred using the UPGMA method. The number in parentheses indicates accession number of endophytic fungus obtained from GenBank database. *Indicates fungus under study.

metabolites production in endophytic fungi may take place below normal room temperature (35°C). At low (15°C) and high temperature (45°C) little growth or metabolites production was observed. pH 6 was optimal for growth and metabolite production with little growth at pH 3 and no growth or metabolite production at pH 10. Many fungi have been reported to grow in acidic conditions but at very low pH maximum toxic metabolites are produced which could adversely affect the growth and metabolites production (Digrak & Eluk 2001). Maximum growth and metabolite production occurred after 10–12 days of incubation, thereafter growth and metabolite production gradually decreased. This agrees with other studies of endophytes where optimum metabolites have been recorded after 2 weeks of incubation.

In our study the metabolite produced by *F. solani* was identified as volatile hydrocarbons. Volatile hydrocarbons meta-bolites have also been reported in some endophytic fungi with antimicrobial activity against human and plant pathogenic bacteria and fungi (Strobel et al. 2001, Stinson et al. 2003). To date endophytic fungi from yew species have been reported to produce taxol, the same compound produced by the host. But, ongoing search for new metabolites from yew species has led to discovery of new compounds, namely azaphilone and two azaphilones (Li et al. 2010). This clearly indicates that yew endophytes can produce various metabolites, which the host could also produce but there is little work in this direction. The present investigation is the first report of an endophytic *F. solani* isolated from *T. baccata* producing volatile hydrocarbons with antimicrobial activity. The production of volatile metabolites similar to hydrocarbons indicate that further investigation on this fungus for biofuel should be undertaken as recently reported from an endophytic fungus, *Gliocladium roseum*

(Strobel et al. 2008). Our finding also emphasizes that *T. baccata* could be explored for hydrocarbons for fuel production.

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