

Research Article



Antimicrobial and Siderophore Activity of the Endophytic Fungus *Acremonium sclerotigenum* Inhabiting *Terminalia bellerica* Roxb.

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ABSTRACT

An endophytic fungus *Acremonium sclerotigenum* was isolated from the leaves of *Terminalia bellerica* and screened for siderophore production and antimicrobial (antibacterial, antifungal) activity. The endophyte was identified as *Acremonium sclerotigenum* based on 18S rDNA sequence analysis. The crude ethyl acetate extract showed potent antimicrobial activity against all test pathogens including gram positive (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*) and gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria. Inhibition zones were more than 10 mm against *E. coli*, *B. subtilis* and *B. cereus*. The crude compound also showed siderophore activity.

Keywords: *Acremonium* sp., Endophytic fungus, Antimicrobial activity, Siderophore activity

INTRODUCTION

Endophytic microorganisms are a significant source of novel bioactive secondary metabolites.^{1,2} The ratio of novel metabolite structures from soil isolates and endophytes, and found 51% new structures from endophytes compared to 38% in soil fungi.³ Endophytic microorganisms produce secondary metabolites of biotechnological interest, including antibiotics, agrochemicals and chemotherapeutic agents to human welfare.⁴ Generally plants growing in unique environmental settings having special ethnobotanical uses or interesting endemic locations possess novel endophytic microorganisms which can supply new leads.⁵ One such special habitat in southern India, a deciduous forest of Bhadrachalam of newly formed Telangana state is at the verge of disappearance due to the ongoing Polavaram irrigation project that was given national status in recent times.

Terminalia bellerica is a deciduous tree of immense medicinal value belonging to family Combretaceae is growing widely in Bhadrachalam forests of Telangana, India and also other parts of Sri Lanka and South East Asia. It is used in the Traditional system of medicine like Ayurveda, Siddha and Unani and one of the three fruit drug mixture, Triphala of herbal medicine.

Bhadrachalam forest spread along the river Godavari in the state of Telangana, India is basically a dry deciduous forest with local pockets of moist deciduous forest elements which provide a special environment for endophytic fungi to diversify. The threat of submergence by the ongoing Polavaram project necessitates the assessment of plant endophyte microbial diversity.

We report the frequently isolated fungal endophyte *Acremonium sclerotigenum* from *T. bellerica* from Bhadrachalam forest and broad spectrum antimicrobial activity of the secondary metabolites in this paper.

MATERIALS AND METHODS

Collection of Plant Samples

Terminalia bellerica was collected from the Bhadrachalam forests, Khammam district, Telangana. The samples were collected using the paper bags or perforated poly bags to avoid desiccation of plant material, and processed to isolate the endophytic fungi.

Isolation of Endophytic Fungi

The freshly collected samples were washed thoroughly in running stream of tap water to remove the dust particles present on the surface of leaves. The leaf samples were cut into 2-5 mm segments and were surface sterilized by sequentially dipping into 0.5% sodium hypochlorite (2 min) and 70% ethanol (2 min), and rinsed with sterile water, then allowed to surface-dry under sterile conditions.⁶ The leaf pieces were placed on sterile blotting paper to drain the excess water and dried under laminar airflow chamber. The surface sterilized segments were transferred to Petri dishes containing Potato Dextrose Agar (PDA), Malt Extract agar (MEA) and Water Agar medium (WA). The Petri dishes were sealed with parafilm and incubated at room temperature, until fungal growth appeared. Fungal colonies growing in the petri dishes were transferred to fresh PDA slants.

Identification of the Endophyte

The endophytic fungal strain (OU E- 211) was identified based on morphology of the fungal culture colony, the characteristics of the spores, and reproductive structures using standard identification manuals⁷ and characterized with molecular methods.

Molecular Identification of Endophytic Fungi

Fungal strain (OU E- 211) was characterised by amplifying the 18S rDNA using universal primers ITS 1 (with base sequences TCCGTAGGTGAACCTGCGG) and ITS 4 (with



base sequences TCCTCCGCTTATTGATATGC).⁸ The rDNA was sequenced and the sequencing result obtained was pasted in Basic Local Alignment Search Tool (BLAST) software supported by National Institute of Health (NIH) using the National Centre for Biotechnology Information (NCBI) database (<http://www.NCBI.NLM.NIH.GOV>) to compare and align with the sequences of known fungi. The rDNA sequence was deposited with NCBI.

Extraction of Fungal Metabolites

The endophytic fungus, *Acremonium* was grown on Rice medium for the extraction of secondary metabolites. It was carried out by transferring fresh fungal cultures into Erlenmeyer flasks (1L each) containing 100 g rice media for solid cultures. The cultures were then incubated at room temperature for 30 days (No shaking). Rice medium consists of 100 g rice in 100 ml distilled water.

After incubation period, 250 ml ethyl acetate (EtOAc) was added to each flask with culture and left overnight. Culture media were then cut into pieces to allow complete extraction and left for 3–5 days. Then filtration was done followed by repeated extraction with EtOAc. The solvent phase and aqueous phase was separated by using separating funnel. Solvent phase was collected and condensed using rotary vacuum evaporator. The dry residue was redissolved in ethyl acetate for further tests.

Antimicrobial Activity

Test organisms include five bacterial and three fungal pathogens. Gram-positive bacteria: *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus* and Gram-negative bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*. The plant pathogens are *Fusarium oxysporum*, *Colletotrichum dematium* and *Macrophomina phaseolina* were evaluated for the antimicrobial activity.

Antibacterial Assay

An aliquot of 100 µl of bacterial liquid culture, from exponential growth phase, was spread on to the surface of Luria Bertani (LB) agar plate. Immediately, 10 µl of test samples were loaded onto the paper disc to give final disc loading concentrations of 250-1000 µg for crude extracts. The impregnated discs were placed onto the surface of LB plates previously seeded with the selected test organisms, along with discs containing solvent blanks. The culture was then incubated at 28-30 °C for 24-48 h. After incubation, the diameter of the inhibition zone was measured in millimeters.

Antifungal Assay

Antifungal activity of crude secondary metabolites was tested by agar well method. The sterilized Potato dextrose agar medium (PDA) was poured in to a Petri dish in a uniform thickness and kept aside for solidification. Using sterilized swabs, even distribution of lawn culture was prepared using desired fungi such as *F. oxysporum*, *C. dematium*, and *M. phaseolina* in PDA plates. Using sterile well cutter three wells were made in plates at uniform

distance. Different concentrations of solvents extracted fungicidal compound was added in to the wells. The plates were incubated at room temperature for 48 hours. After incubation, the zone of inhibition was recorded.

Siderophore Assay

Siderophore production was detected by using chrome azurol S (CAS) assay developed by Schwyn and Neilands (1987).⁹ the medium contains an iron CAS-HDTMA (Hexadecyltrimethyl ammonium bromide) complex which is blue coloured. The presence of iron chelator (Siderophore) is indicated by decolourization of the blue coloured ferric-dye complex, resulting in a yellow halo around the colonies. Chrome azurol S agar medium plates were prepared and spot inoculated with endophytic fungal crude compounds and incubated at 28 °C for 48-72 h. Development of yellow to orange halos around the compound was considered positive for Siderophore production.

RESULTS



Figure 1: Colony morphology of *Acremonium* sp.

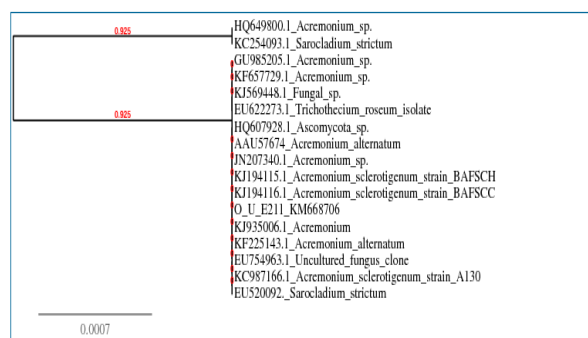


Figure 2: Phylogenetic relationships of *Acremonium* sp.

The medicinal tree, *T. bellerica* harboured sixteen endophytic fungi in its foliage. The fast growing white, fluffy colonies were predominant and frequently isolated along with mycelia sterilia. The fungus showed long awl-shaped phialides producing cylindrical, one-celled conidia mostly aggregated in slimy heads at the apex of each phialide with a basal septum and was identified as a species of *Acremonium* (Fig. 1). To confirm and to exactly identify the endophyte OU E- 211 up to species level, 18S rRNA characterization was done with universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC). The rRNA sequences were analysed by BLAST analysis for alignment and compared with NCBI database and phylogenetic tree analysis. The 18S ribosomal RNA gene (569 base pairs) of endophytic fungal

isolate showed 99% similarity with endophytic fungus *Acremonium sclerotigenum* (GenBank No. AY KJ194116.1). The 18S rRNA sequence is deposited in NCBI database and the accession number given is **KM668706** (Fig. 2).

Table 1: Antibacterial activity of crude extracts obtained from solid state fermentation endophytic fungus *Acremonium* isolated from *Terminalia bellerica*.

Microorganism	Zone of inhibition (mm)		
	<i>A. sclerotigenum</i> (OUE 211)		
	Concentration (µg /ml)		
	250	500	1000
<i>Bacillus cereus</i>	6.5	5.5	10
<i>Bacillus subtilis</i>	6.0	7.0	11
<i>Escherichia coli</i>	5.5	7.5	12
<i>Staphylococcus aureus</i>	4.0	6.0	8.0
<i>Pseudomonas aeruginosa</i>	3.0	3.5	4.5

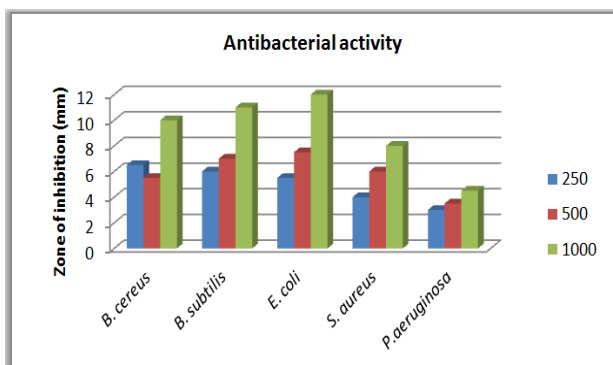


Figure 3: Antibacterial activity of secondary metabolites obtained from solid state fermentation of endophytic fungus *Acremonium sclerotigenum* isolated from *Terminalia bellerica*

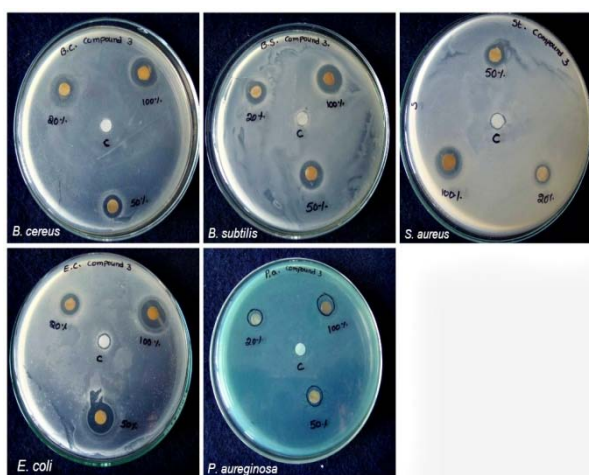


Figure 4: Anti bacterial activity secondary metabolites obtained from endophytic fungus *Acremonium sclerotigenum* isolated from *Terminalia bellerica*.

Antimicrobial Activity

The secondary metabolite extract of the endophyte was antibacterial in nature as it inhibited gram positive and gram negative bacterial pathogens (Table 1). The zone of

inhibition was in the range of 3.0 to 12 mm diameter. Plant pathogenic *Fusarium* and *Colletotrichum* were also inhibited by the crude extracts of *A. sclerotigenum* (Fig. 3, 4). The inhibition against *F. oxysporum* was more at high concentration while *C. dematium* was inhibited even at low concentrations (Fig. 5).

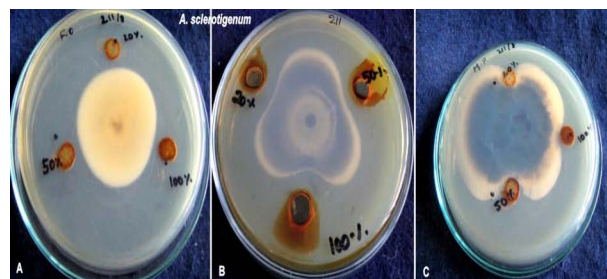


Figure 5: Antifungal activity of crude compound obtained from solid state fermentation of endophytic fungus *Acremonium* isolated from *Terminalia bellerica* (A= *F. oxysporum*; B= *C. dematium*; C= *M. phaseolina*).

Siderophore Production

The secondary metabolites obtained from solid state fermentation of endophytic fungus *Acremonium* isolated from *Terminalia bellerica* shows the positive for siderophore activity (iron chelating) (Fig. 6).

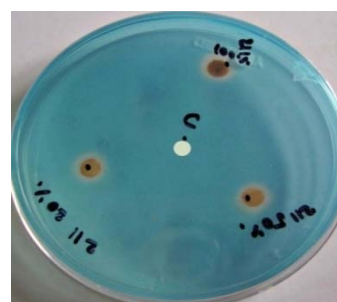


Figure 6: Siderophore productions of secondary metabolites obtained from endophytic fungus *Acremonium sclerotigenum* isolated from *Terminalia bellerica*

DISCUSSION

Acremonium species are frequently identified as endophytes of many plant species^{10,11}. Endophytic association of *Acremonium* sp. has been reported from a wide range of hosts like *Festuca grass* of North America¹² tropical forage grass, *Brachiaria brizantha*¹³, *Taxus globosa*¹⁴ and *Sesbania grandiflora*.¹⁵ *Acremonium* sp. has also been isolated as an endophyte from the roots of traditional Chinese medicinal herb, *Macleaya cordata* and its extracts exhibited strong inhibition on test bacteria¹⁶. Petroski have shown that species of *Acremonium* increased alkaloid production in its host, *Stipa robusta*.¹⁷ In this paper we report for the first time, the endophytic association of *Acremonium* from *Terminalia bellerica* an important medicinal plant of tropical deciduous forests of Telangana state, India. *Acremonium* species of along with mycelia sterilia dominated the endophytic fungi of Egyptian medicinal plants. Isolates of *A. strictum* from

different hosts have shown significant difference in biological activity.¹¹ Our results show not only antibacterial nature of the crude extract of *Acremonium sclerotigenum* and also the ability to inhibit plant pathogenic fungi like *F. oxysporum* and *C. dematium*. Earlier Strobel recorded antifungal activity by *Acremonium* sp. isolated from *Taxus baccata*. Broad spectrum antimicrobial activities of the crude extracts of *A. sclerotigenum* suggest the potential of this endophyte for bioactive compounds involved in the plant-endophyte relationship.¹⁸ Antifungal nature and the ability of *A. sclerotigenum* to produce siderophores gives this endophyte an added advantage to be a potential biocontrol agent of plant pathogens.

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REFERENCES

1. Tan RX and Zou WX, Endophytes: a rich Source of functional metabolites. Nat. Prod. Rep, 18, 2001, 448-459.
2. Strobel G, Daisy B, Castillo, Harper J, Natural products from endophytic microorganisms. J. Nat. Prod, 67, 2004, 257-268.
3. Schulz B, Boyle C, Draeger S, Römmert A-K, Krohn K, Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological Research, 106, 2002, 996-1004.
4. Padhi L, Kishore MY, Panda KS, Endophytic fungi with great promises: A Review. J. Adv. Pharm. Edu. & Res, 3 (3), 2013, 152-170.
5. Strobel GA, Endophytes as source of bioactive products. Microbes and infection, 5, 2003, 535-544.
6. Nithya K, Muthumary J, Bioactive metabolite produced by *Phomopsis* sp., an endophytic fungus in *Allamanda cathartica* linn. Recent Res. Sci. Technol, 3(3), 2011, 44-48.
7. Barnett HL, Hunter BB, Fourth edition Illustrated genera of imperfect fungi. American Phytopathological Society, 1998.
8. White TJ, Bruns T, Lee, Taylor J, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (Eds.), PCR protocols: a guide to methods and applications. Academic Press, New York, USA, 1990, 315-322.
9. Schwyn B, and Neilands JB, Universal Chemical Assay for the Detection and Determination of Siderophores. Analytical Biochemistry, 160, 1987, 47-56.
10. De Almeida MN, Guimaraes VM, Bischoff KM, Falkoski DL, Pereira OL, Goncalves D, De Rezende ST, Cellulases and hemicellulases from endophytic *Acremonium* species and its application on sugar-cane bagasse hydrolysis. Applied Biochemistry and Biotechnology (In Press), 2011.
11. Selim KA, El-Beih AA, AbdEl-Rahman TM, El-Diwany AI, Biodiversity and anti microbial activity of endophytes associated with Egyptian medicinal plants. Mycosphere, 2(6), 2011, 669-678.
12. An ZQ, Liu JS, Siegel MR, Bunge G, Schardl CL, Diversity and origins of endophytic fungal symbionts of the North American grass *Festuca arizonica*. Theor. Appl. Genet. 85, 1992, 366-371.
13. Kelemu S, White JF, Munoz F, Takayama Y, An endophyte of the tropical forage grass *Brachiaria brizantha*: isolating, identifying, and characterizing the fungus, and determining its antimycotic properties. Can. J. Microbiol, 47, 2001, 55-62.
14. Soca-Chafre G, Rivera-Orduna FN, Hidalgo-Lara ME, Hernandez-Rodriguez C, Marsch R, Flores-Cotera LB, Molecular phylogeny and paclitaxel screening of fungal endophytes from *Taxus globosa*. Fungal Biol, 115, 2011, 143-156.
15. Powthong PB, Jantrapanukon A, Thongmee and Suntornthiticharoen P, valuation of endophytic fungi extract for their ant microbial activity from *Sesbania grandiflora* (L.) Pers Int J PharmBioed Rs, 3, 2012, 132-136.
16. Shan T, Jingfeng Lou, Shan Gao, Yufan Zhou, Weibo Sun, Chao Luo and Ligang Zhou Antibacterial activity of the endophytic fungi from a traditional Chinese herb *Paris polyphylla* var. *chinensis* African Journal of Microbiology Research, 6(14), 2012, 3440-3446.
17. Petroski RJ, Powell RG, Clay K, Alkaloids of *Stipa robusta* (sleepy grass) infected with an *Acremonium* endophyte. Nat. Toxins, 1, 1992, 84-88.
18. Strobel GA, Torczynski R, Bollon A, *Acremonium* sp.—a Leucinostatin. A producing endophyte of european yew (*Taxus baccata*) Plant Sci., 128, 1997, 97-108.

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