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Isolation and bioactive potential of fungal endophytes associated with medicinal plant *Acmella paniculata* (Wall. ex DC.)R.K.Jansen

UDIPTA DAS *[1], JYOTISHMOYEE BORUAH[1], CHANDAN RAY BARMAN[3], PANNA DAS[2] AND AJAY KRISHNA SAHA[1]

[1]*Mycology and Plant Pathology Laboratory, Department of Botany, Tripura University, Suryamaninagar, Tripura -799022*

[2]*Microbiologyy Laboratory, Department of Botany, Tripura University, Suryamaninagar, Tripura -799022*

[3]*Department of Pediatrics, Gouri Devi Institute of Medical Sciences and Hospital, Durgapur 713212, West Bengal*

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Acmella paniculata (Wall. ex DC.)R.K.Jansen (Syn.: *Spilanthespaniculata*) belonging to Family Asteraceae is an important medicinal plant. The present investigation focused on isolation and characterization of fungal endophytes from *A. paniculata* and selected isolates were screened for their antimicrobial activity. A total of 171 isolates of endophytic fungi assigned to 10 representative morphotypes, including six genera i.e. *Aspergillus, Trichoderma, Curvularia, Diaporthe, Penicillium,* and *Fusarium* were found and three Mycelia Sterilia forms were also obtained from leaves, stems and roots of *A. paniculata.* Endophytic colonization frequency was found to be higher in leaves (97.14%) as compared to stem (87.14%) and root (60%) samples. Antibacterial activity was determined using the agar disk diffusion method against the gram-negative strains *E.coli*, *P. aeruginosa* and gram-positive *B. subtilis*, *S. aureus* with inhibition zone diameter ranging from 7.0-19.4 mm. Among the tested fungal strain endophytic fungi *Penicillium* sp. isolated from the leaves of *A. paniculata* exhibited the strongest antimicrobial activity against *E.coli* with inhibition zone of 19.3±0.4 mm.The outcome of the study is providing scientific evidences for endophytic fungi associated with medicinal plant *A. paniculata* and various endophytic fungi could be exploited as sources of novel natural antimicrobial products.

Key words: Endophytes, *Acmella paniculata,*medicinal plant, Asteraceae, Ascomycota, antimicrobial activity

INTRODUCTION

Endophytes are microorganisms that are found to be inherent in the core tissues of plants without causing any disease in the host. But there are chances that they may cause disease after their latency period. Endophytes colonize plant tissues internally without any symptoms so they are called asymptomatic. Endophytes live in a symbiotic relationship with the host. They show variation in host-microbe interaction and are known to provide nutritional benefits and protection against environmental and microbial stresses (Schulz and Boyle, 2005). Endophytic fungi provide several beneficial roles to the host plant. They enhance the growth and vigour of their host plants through acquiring different nutritional elements such as nitrogen and phosphorus useful for plants (Hartley and Gange, 2009). Some endophytic fungi could also enhance thegrowth and fitness by increasing different plant hormones. Endophytic fungi play a vital role in increasing the resistance of different environmental stresses of host plant by producing various bioactive compounds such as alkaloids, terpenes, flavonoids etc. (Firakova *et al.,* 2007).

Acmella paniculata (Wall. ex DC.) R. K. Jansen (Syn.- *Spilanthes paniculata*) belonging to family Asteraceae is a significant medicinal plant distributed in the tropical and subtropical regions all over the world with a wealthy source of beneficial and medicinal constituents. Commonly, the plant is

*Correspondence : udiptadas93@gmail.com

known as "toothache plant" which reduces tooth pain. Some other important conventional uses of this plant are the treatment of rheumatism, as a sialagogue for stammering, tongue paralysis, antipyretic, sore throat, and gum infections (Ley *et al.*,2006). *Acmella paniculata* has multiple pharmacological actions such as Local anaesthetic Activity, Anti-Inflammatory/Analgesic Activity, Antifungal activity etc. (Dubey *et al.*, 2013). *Acmella* contains many biologically active compounds (Prachayasittikul *et al.*, 2009), of which the most studied have been the alkylamides, which this plant possesses in abundance. Some common alkylamides compounds isolated from *Acmella paniculata* are spilanthol or affinin, amide derivatives etc. (Prachayasittikul *et al.*, 2013).

The literature survey revealed that till date there are very few experimental studies accomplished regarding endophytic fungi of *Acmella paniculata*. Hence the present study is focused on isolation and characterization of fungal endophytes from *A. paniculata* and selected isolates were screened for antimicrobial activity.

MATERIALS AND METHODS

Plant sample collection

Leaf, stem and root samples were collected from apparently healthy and wild plant *A. paniculata* from Suryamaninagar, Tripura (N 23°45'30" E 91°15'50" at 23m). All plant samples were kept in plastic bags and brought back to the Mycology and Plant Pathology Laboratory at Tripura University. The plant sample was preserved in the herbarium centre Department of Botany, Tripura University after identification having collection No. 2730(Figs.1 & 2).

Media preparation

Malt extract agar (MEA) medium is used for the isolation of fungal endophytes. An aliquote of, 50-µg/ml streptomycin (Hi-media) was added to avoid bacterial contamination.

Surface sterilization for isolation of endophytic fungi

Surface sterilization of plant segments were done within a few hours of sample collection. Plant tissues (leaf, stem, roots) were surface sterilised by the procedure mentioned by Tan *et al.*, 2012. With a few modifications, the procedure can be briefly described as all samples were carefully washed under tap water then sterilised by immersing 70% ethanol for 30 sec and 3% sodium hypochlorite (NaOCl) solution for 5 min then all plant tissues were rinsed three times with sterile distilled water and surface-dried with sterile blotting paper.

Isolation and cultivation of endophytic fungi Isolation and cultivation of endophytic fungi is done by the method described by Tan et al., 2012. The sterilized plant segments were excised into pieces with dimension of 0.5 × 0.5 cm using a sterile blade and forceps. The fragments were aseptically transferred to previously prepared Petri dishes containing Malt extract agar medium supplemented with streptomycin to inhibit the growth of bacteria. The Petri dishes were incubated at 25° ± 2°C for 5days at BOD incubator. Colonies that emerged from the cultures were subcultured in fresh MEA medium maintain the conditions for purification.

Identification of fungal endophytes

The fungal strains were identified by observing morphologically and microscopically. Morphological identification of fungi was obtained through the study of colony appearance, fungal morphology, mycelium structure and colour. Microscopically (OLYMPUS-CX21i) the fungi were identified by staining the fungus with lactophenol cotton blue and comparing with the help of standard textbook and online portals (Ellis, 1971; Domsch *et al.*, 1980; Watanabe, 2002 and www.mycobank.org).

Antimicrobial screening of selected fungal endophytes

Preparation of fungal extract

The fungal extract was prepared by following the method of Nath *et al.*, 2012. Fungal strains were grown on malt extract broth medium by inoculating them with mycelial plugs (3mm) from actively growing pure culture. The agar plugs were placed in 250 ml conical flasks containing 100 ml malt extract broth medium. The flasks were incubated for 3 weeks under 25± 20C in shaking condition at 150 rpm. The cultured broth was filtered to separate the fungal mycelia and the filtrate. The culture filtrate was filtered with an equal volume of methanol twice and it was evaporated under reduced pressure using a rotary vacuum evaporator (ROTAVAP model number: PBV-7D) for removal of solvent and

appearance of dried powder. The dried fungal extracts were dissolved in Dimethyl sulfoxide (DMSO) (1 mg per ml) and evaluated their antimicrobial activity by agar disc diffusion method (Heatley, 1944).

Antimicrobial assay was performed by well-known Agar disc diffusion method in Nutrient agar media (Heatley, 1944). In this procedure suspensions of the test microorganisms were inoculated onto the agar plates, spread uniformly and the inoculum was allowed to dry for a few minutes. Then filter paper discs (~ 6mm in diameter) were loaded with the extracts. The loaded discs were placed on the surface of the agar medium. The plates were kept for incubation under suitable conditions. Streptomycin was used for positive control and Dimethyl sulfoxide (DMSO) for negative control. At the end of incubation, inhibition zones formed around the disc were measured. These studies were performed in triplicate.

Test organism

The bacterial species used for antimicrobial test of fungal extract were the gram-negative *Escherichia coli* (MTCC-40), *Pseudomonas aeruginosa* (MTCC-424) and gram-positive *Bacillus subtilis* (MTCC-619), *Staphylococcus aureus* (MTCC-96). The bacterial strains were provided by the Institute of Microbial Technology (IMTech), Chandigarh, India.

Data analysis

Colonization rates (CR%) of fungal isolates were calculated as follows (Tan *et al.,* 2018):
where, Nsc = Number of segments infected by fungi,

$$CR\% = \left(\frac{Nsc}{Nss}\right) X100$$

Nss = Total number of segments investigated.

Isolation rates (IR) of fungal isolates were calculated as follows: (Tan *et al.*, 2018):
where Ni = number of segments from which fungal

$$IR\ \% = \left(\frac{Ni}{Nt}\right) X100$$

species were isolated, Nt = total number of segments incubated.

Statistical analysis

All samples determinations were conducted in triplicate. Statistical analysis was done with the help of Microsoft Office Excel 2007. The results were expressed as mean values ± standard deviations (SD).

RESULTS AND DISCUSSION

Isolation of Endophytic fungi

Endophytic fungi were the most unexplored organism with a rich source of biodiversity and biological product. In the present study, endophytic fungi were isolated from different plant tissues (leaf, stem and roots) of *A. paniculata*. Each of 70 leaf, stem and root samples were sterilized for endophytic fungal isolation. These tissue segments leads to a total of 171fungal colonies recovered from 210 segments. The colonization rates and isolation rates for endophytic fungi are given below (Table1 and Table2). The Isolation rate was significantly higher in the leaves of *A. paniculata*.The present data showed that *A. paniculata* exhibited a rich diversity of endophytic fungi, and we found that the most ubiquitous phylum is Ascomycota, which is reportedly among the most prevalent group of eukaryotes (Fig.3 and Fig. 4). In this present study leaves samples of examinedplant colonized highest endophytic diversity as compared to stem and root samples.Similar findings were reported by Weber and Anke (2006); Gamboa and Bayman (2001) and Kurandawad and Lakshman (2014).Thus, the present finding has supported that the endophytes isolated from leaf samples exhibited greater diversity and high colonization frequency compared to the endophytes of the other plant parts examined.

According to their morphological characteristics on malt extract agar, and all cultured endophytic fungi were identified on the basis of microscopic observations (morphology of vegetative and reproductive structures). The 171colonies from different plant parts were assigned to 10 representative morphotypes, including six genera i.e. *Aspergillus, Trichoderma, Curvularia, Diaporthe, Penicillium,* and *Fusarium* sp. and three sterile forms were found. The colonization frequencies of the fungal isolates from *A. paniculata* were from 2.72 % to 33.33%.Nonsporulating endophytic fungal strain, which failed to sporulate in culture condition was designated as sterile hyphae. This is the common

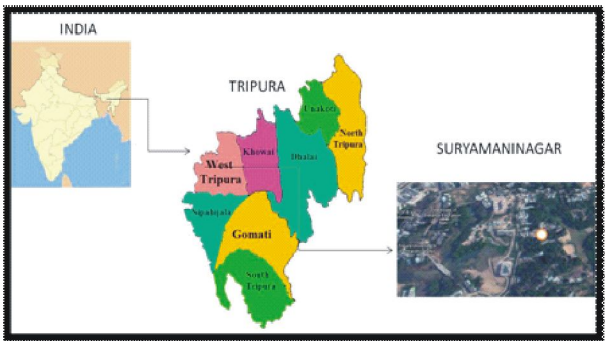

Fig. 1 : Collection site of plant samples for isolation of endophytic fungi

Fig. 2 :Adult plant of *Acmellapaniculata*(A), Habitat of the plant (B) and Herbarium(C)

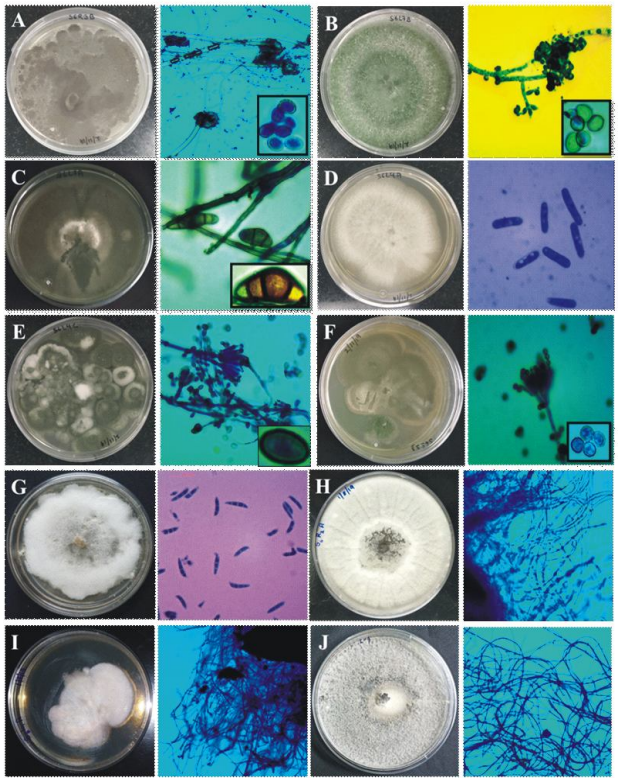

Fig.3: Pure cultures and microscopic study of endophytic fungal isolates: **A.** *Aspergillus sp.* (S6R3B) **B**. *Trichoderma* sp. (S6L7B) **C**. *Curvularia* sp. (S6L7A) **D**. *Diaporthe* sp. (S6L4A) **E**. *Penicillium* sp. *1*(S6L4C)**F**. *Penicillium* sp. *2* (S6S3D)**G.** *Fusarium* sp. (S6S3C) **H-J.** Some non-sporulating fungal mycelia

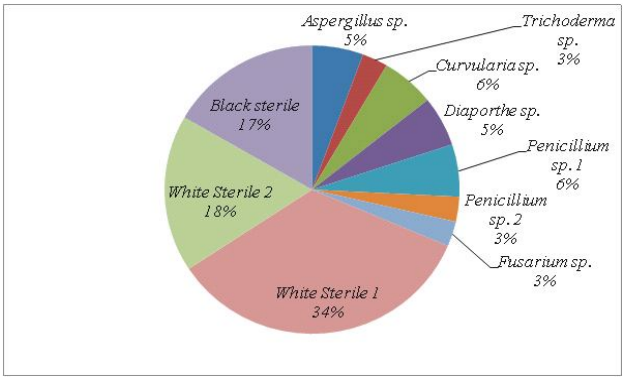

Fig.4: Culturable endophytic fungi from *A. paniculata* and corresponding isolation rates (IR%).

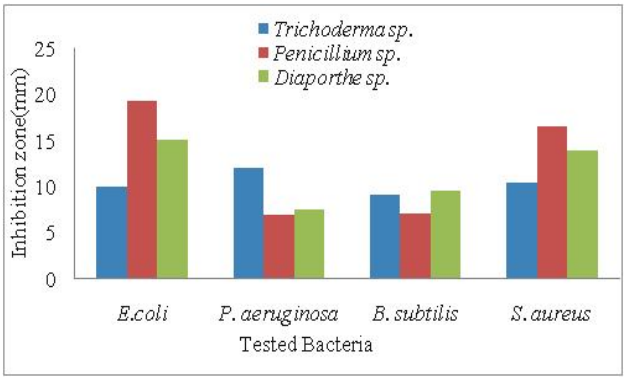

Fig.5: Antibacterial activity of crude extracts of endophytic fungi.

Table 1: Colonization rate of Endophytic fungi from *A. paniculata*

Tissues	Segments Examined	Segments infected	Total CR%
Leaf	70	78	97.14
Stem	70	61	87.14
Root	70	42	60
Total	210	171	

Table 2: Culturable endophytic fungi from *A. paniculata* and corresponding isolation rates (IR%).

Lab. Accession number	Fungal isolates	Isolation plant tissue	IR %
S6R3B	*Aspergillus sp.*	Root, leaf	5.45
S6L7B	*Trichoderma sp.*	Leaf	2.72
S6L7A	*Curvularia sp.*	Leaf, stem, root	5.71
S6L4A	*Diaporthe sp.*	Leaf, stem, root	5.45
S6L4C	*Penicillium sp.1*	Leaf	5.71
S6S3D	*Penicillium sp.2*	Stem	2.72
S6S3C	*Fusarium sp.*	Stem	2.72
S6R2A	White Sterile 1	Leaf, stem, root	33.33
S6L3B	White Sterile 2	Leaf, stem, root	17.14
S6R3D	Black sterile	Leaf, stem, root	16.14

Table 3: Antibacterial activity (inhibition zone mm) of crude extracts of endophytic fungi

Fungal taxa	Lab accession number	Inhibition zone in diameter on Petri plates (mm)			
		E.coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
Trichoderma sp.	S6L7B	10.1±0.6	12.1±0.2	9.1±0.2	10.5±0.4
Penicillium sp.	S6L4C	19.3±0.4	7.0±0.0	7.1±0.2	16.5±0.4
Diaporthe sp.	S6L4A	15.1±0.6	7.6±0.2	9.6±0.2	13.9±0.2

problem relating to endophytic fungal identification (Gamboa and Bayman, 2001; Promputtha *et al.*, 2005). In the present study, sterile spp. were found to be the most dominant endophytic species which were not organ-specific, this results came in accordance with many endophytic studies. (Froehlich *et al.*, 2000; Promputtha *et al.*, 2005).

Antimicrobial essay

Microorganisms produce many bioactive compounds as secondary metabolites including antibiotics and cytotoxic compounds. Antimicrobial activity of the filamentous fungal extracts against bacterial pathogen strains was determined by agar disc diffusion method. We have selected three fungal isolates for antimicrobial screening. The three selected fungi identified as *Trichoderma* sp., *Penicillium* sp. and *Diaporthe* sp. All extracts showed antimicrobial activity against the test organisms i.e. gram-negative strains *Escherichia coli*, *Pseudomonas aeruginosa* and gram-positive *Bacillus subtilis*, *Staphylococcus aureus* with inhibition zone diameter ranging from 7.1-19.3 mm (Table. 3 and Fig.5). Extract of *Trichoderma* sp. showed the highest activity against *P. Aeruginosa* with inhibition zone 12.1±0.2 mm and lowest against *B. subtilis* with inhibition zone diameter of 9.1±0.2mm. Whereas extract of *Penicillium* sp. showed the highest inhibition zone against *E. coli* with inhibition zone of 19.3±0.4 mm and lowest activity against *P. aeruginosa* with inhibition zone 7.0±0.0 mm. *Diaporthe* sp. showed the highest activity against *E. coli* with inhibition zone 12.1±0.2 mm and lowest against *B. subtilis* with inhibition zone diameters of 7.6±0.2mm.The most antimicrobial activity was recorded for *Penicillium* sp. (S6L4C), which inhibited the growth of most test organisms; however, *Trichoderma* sp. and *Diaporthe* sp. also had good inhibitory action against test organisms. Thereby, our investigations suggest that these fungal endophytes can be used as producers of metabolites with broad and specific antimicrobial activity. Antibacterial activity may be due to the active components present in the fungal extracts. (Joel and Bhimba,2013).

The outcome of the study has provided scientific evidence for endophytic fungi associated with the medicinal plant *A. paniculata.* Moreover,the present investigation that suggested different endophytic fungi of *A. paniculata* could be exploited as sources of novel natural antimicrobial products,therefore an endeavour humble initiative to explore the potential fungal endophytic population in medicinal plant will pave a opportunities for future fungal endophytic research.

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REFERENCES

Domsch, K.H. Gams, W., Anderson, T.H. 1980.*Compendium of soil fungi.* Volume 1. Academic Press (London) Ltd..

Dubey, S. Maity, S. Singh, M. Saraf, S. A., Saha, S. 2013. Phytochemistry, pharmacology and toxicology of *Spilanthesacmella*: a Review. *Adv. in Pharmacol.Sci.*https://doi.org/10.1155/2013/423750

Ellis, M.B. 1993.*Determination Hypomycetes.* CAB Int.

Firakova, S. Sturdikova, M. and Muckova, M. 2007. Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia*.**62**: 251–257. doi: 10.2478/s11756-007-0044-1

Frohlich, J. Hyde, K.D. Petrini, O. 2000. Endophytic fungi associated with palms. *Mycol.Res*.**104**: 1202-1212.

Gamboa, M. A., Bayman, P. 2001. Communities of endophytic fungi in leaves of a tropical timber tree (*Guareaguidonia*: Meliaceae) 1. *Biotrop*.**33**: 352-360.

Hartley, S. E. and Gange, A. C. 2009. Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. *Annu. Rev. Entomol.* **54**: 323–342. doi: 10.1146/annurev.ento.54.110807.090614

Heatley, N. G. 1944. A method for the assay of penicillin. *Biochem. Jour*.**38**: 61.

Joel, E. L., Bhimba, B. V. 2013. Evaluation of secondary metabolites from mangrove associated fungi *Meyerozyma guilliermondii*. *Alex.Jour. of Med*.**49**: 189-194.

Kurandawad, J. M., Lakshman, H. C. 2014. Diversity of the endophytic fungi isolated from *Acalypha* Indica Linn–A Promising medicinal plant. *Int. Jour. of Sci. and Res. Publ.* : 2250-3153.

Ley, J. P. Blings, M. Krammer, G. Reinders, G. Schmidt, C. O., Bertram, H. J. 2006. Isolation and synthesis of acmellonate, a new un-

saturated long chain 2-ketol ester from *Spilanthe sacmella*. *Nat. Prod. Res.***20**: 798-804.

Nath, A. Raghunatha, P., Joshi, S. R. 2012. Diversity and biological activities of endophytic fungi of *Emblica officinalis*, an ethnomedicinal plant of India. *Mycobiol.***40**: 8-13.

Prachayasittikul, S. Suphapong, S. Worachartcheewan, A. Lawung, R. Ruchirawat, S., Prachayasittikul, V. 2009. Bioactive metabolites from *Spilanthes Acmella* Murr. *Molecules*.**14**: 850-867.

Prachayasittikul, V. Prachayasittikul, S. Ruchirawat, S., Prachayasittikul, V. 2013. High therapeutic potential of *Spilanthes acmella*: a review. *EXCLI jour*.**12**: 291.

Promputtha, I. Jeewon, R. Lumyong, S. McKenzie, E. H. C. , Hyde, K. D. 2005. Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). *Fungal Diversity*. 157-186

Schulz, B. and Boyle, C. 2005. The endophytic continuum. *Mycol. Res.***109**: 661-686.

Tan, X. M. Chen, X. M. Wang, C. L. Jin, X. H. Cui, J. L. Chen, J. , Zhao, L. F. 2012. Isolation and identification of endophytic fungi in roots of nine *Holcoglossum* plants (Orchidaceae) collected from Yunnan, Guangxi, and Hainan provinces of China. *CurrentMicrobiol.***64**: 140-147

Tan, X. Zhou, Y. Zhou, X. Xia, X. Wei, Y. He, L., Yu, L. 2018. Diversity and bioactive potential of culturable fungal endophytes of *Dysosmaversipellis*; a rare medicinal plant endemic to China. *Sci. Rep*.**8**: 5929.

Watanbe, T. 2002.*Pictorial Atlas of Soil and Seed fungi*. 2nd Ed.CRC Press

Weber, R. W. S., Anke, H. 2006. Effects of endophytes on colonization by leaf surface microbiota. Microbial ecology of aerial plant surfaces. CABI International, Cambridge, 209-222.