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Full Length Research Paper

In vitro antimicrobial studies of *Nodulisporium* specie: An endophytic fungus

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***Nodulisporium* sp. an endophytic fungus identified by 28s ribosomal gene sequencing isolated from a medicinal plant, *Nothapodytes foetida* was studied for its *in vitro* antimicrobial activity. Dual culture studies were carried out for antifungal activity where maximum antagonistic activity was against *Alternaria alternata* and *Colletotrichum gleosporoides.* For antibacterial studies, Gram positive and Gram negative human pathogens strains were used. The minimum inhibitory concentration (MIC) of ethyl acetate and methanol fractions of *Nodulipsporium* showed appreciable growth inhibition mainly active against disease causing Gram positive bacteria.**

Key words: *Nothapodytes foetida,* endophytes, antagonism, antimicrobial, *Nodulisporium,* sp., pathogen, bacteria, minimum inhibitory concentration.

INTRODUCTION

Plants commonly act as hosts to a multitude of microbes including parasites, symbionts, endophytes, epiphytes and mycorrhizal fungi (Fisher and Petrini, 1990). These microorganisms may also influence the production of secondary metabolites. Endophytes, the microorganisms that reside in the intercellular spaces of stems, petioles, roots and leave of plants causing no discernible manifestation by their presence have typically gone unnoticed (Strobel and Long 1998). Our search for studying endophyte is driven by the fact that the contribution of the endophytes to the plant may be to provide protection to it by virtue of anti-microbial compounds that it produces. Some of these endophytes may be of interest to agricultural sciences, since they possess anti-fungal, anti-bacterial, anti-malarial and a host of other biological activities.

Here we report an endophyte from the medicinal plant *Nothapodyte foetida*, which grows widely throughout India including north-western Himalayan region. Two naturally occurring alkaloids, Nothapodytines A and Nothapodytines B have been isolated from the stem of *N. foetida* having the antimicrobial property as well (Wu et al., 1996). Moreover *N. foetida* is used as a source of anticancer compound, camptothecin. Several hundreds of compounds with antibiotic activity have been isolated from microorganisms over the years (Harrison et al 1991). The phenomenal success of penicillin led to the search for other antibiotic-producing microorganisms. In the present study, isolated endophytic fungus was also found to possess antagonistic activity against few phyto-pathogens which could be used as potential biocontrol agent in disease management. In addition the endophytic extracts were found active against important Gram positive bacterial pathogens.

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MATERIALS AND METHODS

Isolation and Identification of an endophyte

The endophytic fungus was isolated from twigs of *N. foetida* obtained from the Jodia forest of Karnataka. The organism was isolated by using the method described by (Strobel et al*.,* 1996).

Homology modeling

Total genomic fungal DNA was extracted by cetyl trimethyl

Table 1. Five different test plant pathogens used in dual culture method.

Test pathogen	Disease caused
Penicillium citrinum	Leaf spot, fruit rot, cucumber disease, minor foliar disease.
Asperillus niger	Crown rot, black mold, bole rot, canker.
Drechslera tetramera	Small brown spots.
Alternaria alternate	Black rot, leaf spot, potato early blight.
Colletotrichum gleosporoides	Anthracnose, leaf spot, stem spot.

ammonium bromide (CTAB) method. Briefly the endophyte was grown in 100 ml Sabouraud dextrose broth at 28°C with constant shaking for 5 days. Hundred milligrams of mycelial biomass was taken following washing (two times) with sterile Tris-EDTA (TE) buffer, 6 ml of CTAB extraction buffer and 60 µl of β-mercaptoethanol were added. The mixture was incubated at 65°C for 45 min, and cooled down to room temperature. This was followed by extraction with equal volume of chloroform and centrifuging at 10,000 x g for 10 min. Subsequently, equal volume of isopropanol was added to the supernatant and mixed gently. The obtained DNA pellet was washed with ice cold 70% (v/v) ethanol, vaccum dried and dissolved in 100 µl of TE (pH 8.0).

Small subunit gene sequencing and analysis

The endophytic fungus was identified by the ribosomal gene analysis. The small subunit ribosomal gene was amplified using the D2 LSU Microseq ki (ABI, USA). The amplified products were purified using Microcon columns (Millipore, USA), and sequenced using ABI Prism310 genetic analyzer (ABI, USA) as per the manufacturer`s instructions. The DNA sequence 280 bases (GenBank Acc. No. EU284592) was analyzed for homology studies by BLASTN program (Altschul et al., 1997). The ribosomal gene database (http//ncbi.nim.nih.gov) was accessed and sequence alignment was used as an underlying basis to identify the fungus.

Antimicrobial studies

Dual culture method

This experiment was performed by dual culture method. In this method endophytic fungus was studied for antagonism against fungal strains. Five different test plant pathogens were used for the studies which were obtained from National Fungal Research Institute (NFRI) New Delhi Table 1.

Endophyte and all the test pathogens were grown on Sabouraud's agar plates and incubated at 28°C for seven days. 2 mm dia plugs of newly grown endophyte and test pathogens were taken with the help of cork borer and were aseptically placed 80 mm away from each other on the opposite sides of 90 mm Petri plates containing about 30 ml of fresh Sabouraud's agar medium and incubated at 28°C (Munshi and Dar, 2004). Simultaneously the disc of an endophyte and each test pathogen were placed separately on Sabouraud agar plate which served as control. All the inoculated plates were allowed to grow. Three replicates were used for each pathogen. After few days the plates were observed and growth of an endophyte and test pathogen was measured. The antagonistic activity was analyzed biostatistically. This experiment was carried out thrice.

MIC determination

This method was performed to test endophytic fungus against human pathogens. The chloroform: methanol extract of mycelia (6 g) was prepared. It was subjected to usual silica gel column chromatography and the elution was carried out with benzene, ethyl acetate, methanol to obtain fractions ZPF-1, ZPF-2, ZPF-3 respectively (Wall and Wani 1977). The extracts were tested for antimicrobial activity against human pathogens using microdilution method defined by Clinical and Laboratory Standards Institute (CLSI) formerly known as National Committee for Clinical Laboratory Standards (NCCLS, 1996). A panel of laboratory standard pathogenic strains were used. *Staphylococcus aureus* was obtained from the American Type Culture Collection (Manassas, Va.). Methelene resistant *S. aureus* strains were obtained as a gift sample from Ranbaxy Research laboratories (New Delhi, India). *Escherchia coli, Pseudomonas aeruginosa* from Indian Institute of Integrative Medicines, Jammu. Ciprofloxacin which was used as reference drug was obtained from Cadila Pharmaceuticals, Gujarat India. These pathogenic strains were maintained on Mueller Hinton agar medium at 37°C in stationary phase and subcultured fortnight.

Stock solution of 20 $mgml^{-1}$ was prepared in DMSO. The stock solutions were serially diluted to obtain working test solutions with suitable growth medium. The final concentrations ranged from 4000 to 7.8125 µg/ml for test material and from 0.03 to 64 µg/ml for ciprofloxacin.

Bacterial suspensions were prepared from overnight grown cultures in Mueller Hinton agar medium. The turbidity of the suspensions was adjusted to a McFarland no. 1 in sterile normal saline and was further diluted to 1:50 in Mueller Hinton broth.

100 µl of sterilized growth medium was added in the wells of sterile 96-well plates (U-bottom) from columns 2 to 11. In row A, 200 µl of 2-fold concentrated reference drug solution was added to the wells in column 1, 100 µl was transferred from column 1 to 2, and serially diluted upto 10 column. Column 11 and 12 containing 100 and 200 µl of medium without drug served as growth and medium control respectively. In other rows, test material was processed by same procedure. 100 µl of bacterial suspension was added to the wells of microplates from column 1 to 11 to achieve a final volume of 200 µl per well. Plates were sealed with parafilm and incubated overnight at 37°C. Visually clear well with no growth at bottom of the well was taken as MIC of that particular drug or extract.

RESULTS

Isolation and Identification of endophyte

N. foetida (family: Olacaceae) was chosen as a source plant for isolating the endophyte, since this plant has been reported to be one of the important medicinal plant. Stem twig of plant that is *N. foetida* was used for isolation. Isolated Endophyte typically possesses 3 to 4 µm in diameter which spread as white mat on solid media within 5 to 7 days (Figure 1).

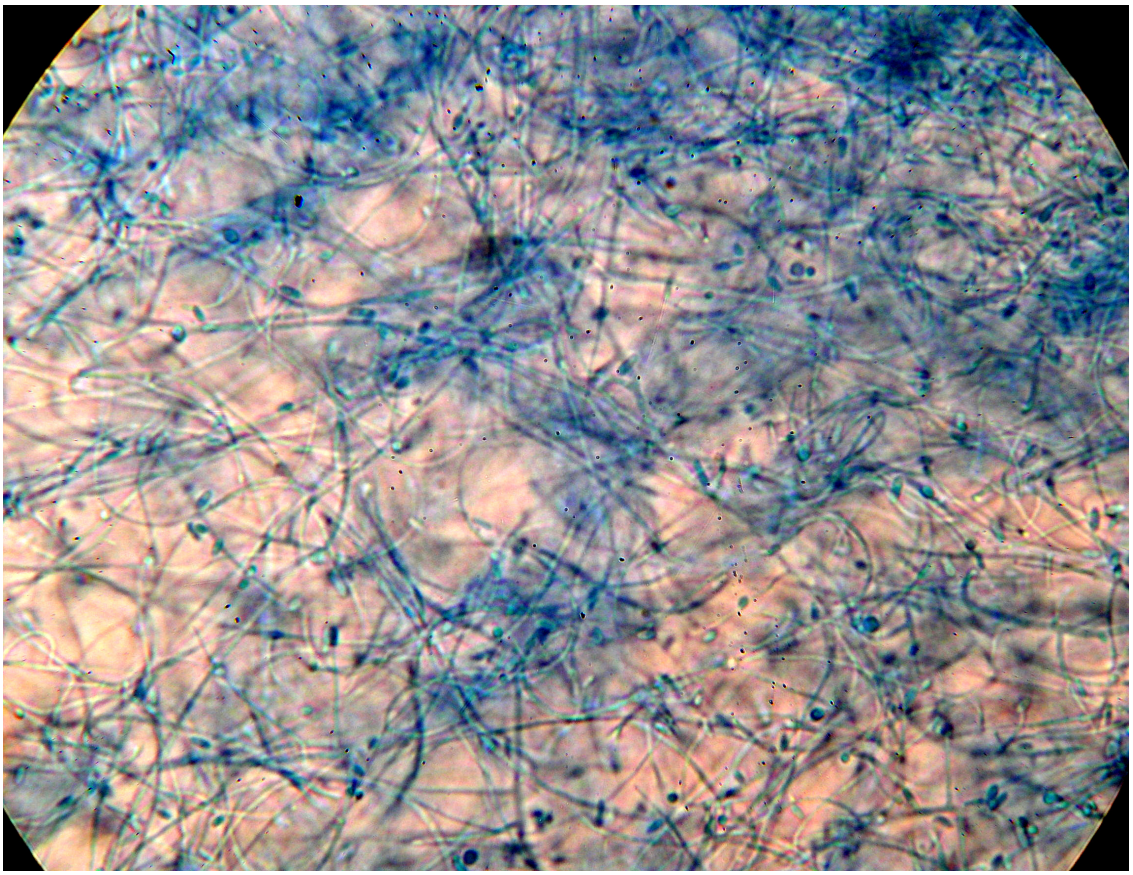

Figure 1. An endophytic fungus from *N. foetida*.

The fungus isolated from the inner bark of *N. foetida* was identified by 28s ribosomal gene sequencing. After 48 h of growth in Sabouraud Dextrose broth (with constant shaking) at 28°C, mycelial biomass could be collected in gram quantities. This collected biomass fraction was further used for DNA isolation, and the pellet obtained using Tris-EDTA buffer was vaccum dried and dissolved in CTAB buffer. Figure 2 shows the distance tree constructed on the basis of homology of 28s ribosomal gene sequence of endophytic strain with close members in GenBank. The isolate showed highest sequence similarity of 95% with *Nodulisporium* sp. CL108.

Antimicrobial studies

Dual culture

In dual culture study, growth of endophyte (*Nodulisporium)* covered the entire medium surface and restricted the growth of all test pathogens (Figure 3). Endophyte inhibited all tested pathogens but maximum antagonistic activity was shown against *Alternaria alternata* and *Colletotrichum gleosporoides,* by covering the entire medium surface. The biostatistical analysis revealed that degree of antagonism against *A. alternata* and *C. gleosporoides*, *Asperigillus niger* and *Penicillium citrinum* is almost similar (Table 2).

MIC determination

Antibacterial activity was determined against human pathogens *S. aureus,* MRSA, *E. coli*, *P. aeruginosa*. The MIC of ethyl acetate and methanol fractions showed appreciable growth inhibition with MIC of 125 and 250 µg/ml against Gram positive strains *S. aureus,* MRSA whereas Benzene fraction did not show any activity. MIC of all fraction against Gram negative microorganism was observed as >4000 µg/ml and not very active. Ciprofloxacin was used as standard antibiotic in this study (Table 3).

DISCUSSION

The endophytic fungus identified as *Nodulisporium* sp. identified by 28s DNA typing was obtained from inner

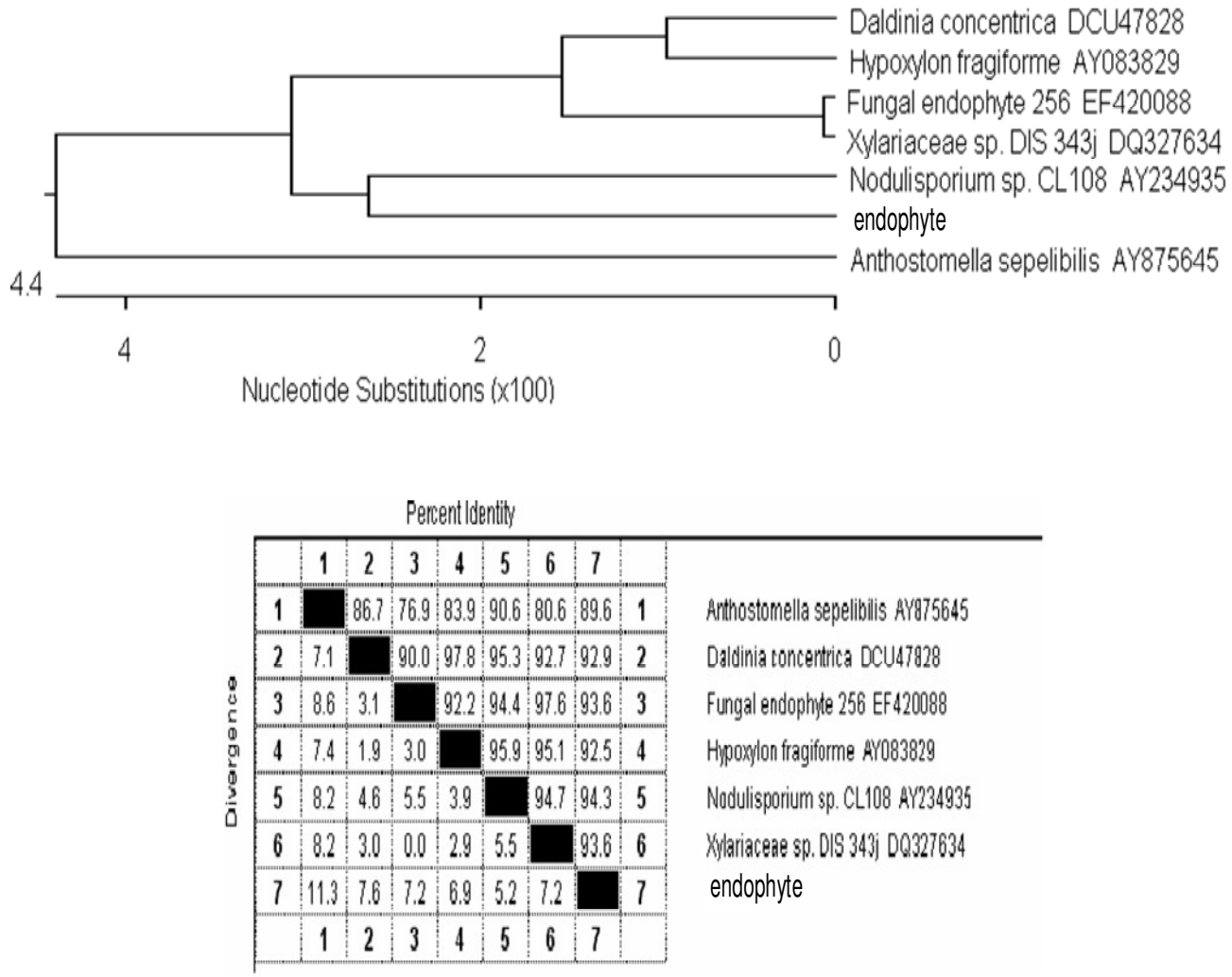

Figure 2. Phylogenetic position of an endophyte.

Table 2. Evaluation of endophyte against different plant pathogens by dual culture method.

Phytopathogens used	Mean growth value of endophyte (mm^2) against pathogens
Penicillium citrinum,	61.33* c
Alternaria alternata	81.66^{NS} a
Colletotrichum gleosporoides	77.66^{NS} a
Drechslera tetramera	68.0** b
Asperigillus niger	61.0* c

(Note- Means followed by similar letter(s) are identical.) NS= Not significant; * = 1%; **= 5%; SEM= 1.77; CD (P=0.05) = 5.57.

bark of *N. foetida* plant collected from Joida forest Karnataka. Until now the only recognized means of controlling plant diseases were to use chemical substances. These methods have attracted huge criticism from environmental groups and thus other means of control have to be investigated. Research on biocontrol through the application of endophytes has the goal of promoting internal fungi from resident to necrotrophic status by stimulating the fungi themselves. Endophytes themselves may also predispose their hosts to environmental damage by reducing the damage threshold. In the present study results of the dual culture test showed that endophytic "*Nodulisporium"* is antagonistic to all the test pathogens. *Nodulisporium* grew rapidly and covered

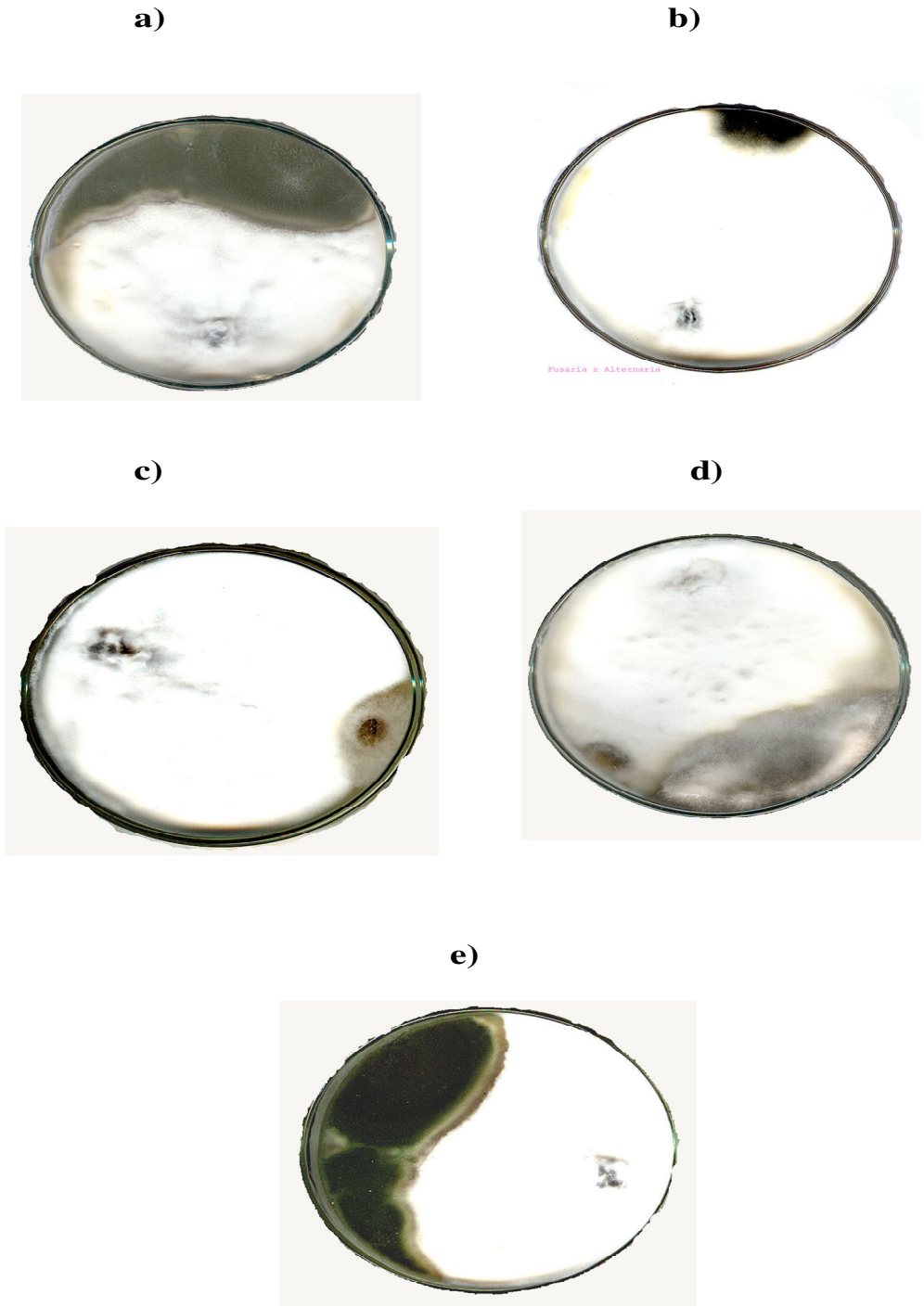

Figure 3. Antagonistic activity of endophyte against (a) *P. citrinum* (b) *A. alternata* (c) *C. gleosporoides* (d) *D. tetramera* (e) *A. niger*

the entire agar surface of Petri dishes after incubation for few days and restricted the growth of all test pathogens but maximum antagonism was shown against *A.alternate* (black rot, leaf spot, potato early blight) and *C. gleosporoides* (anthracnose, leaf spot, stem spot). Therefore, it is interesting to find antagonism in the endophytic fungus isolated from *N. foetida* plant. This may help to emphasize the use of fungus as an important alternate source of biocontrols. The productions of anti-fungal compounds by endophytes have been previously reported by other workers (Liu et al. 2001).

MIC of endophytic fractions were determined against human pathogens. This sensitivity test was performed by microdilution method. The edge of the zone of inhibition

Table 3. MIC determination of organic extract/fractions of fungal mycelia against few bacterial strains.

Fractions from chloroform: methanol (4:1) extract of mycelia	MIC µg/ml			
	S. aureus	**MRSA**	***E. coli***	***P. aeruginosa***
ZPF-1	>4000	>4000	>4000	>4000
ZPF-2	125	125	>4000	>4000
ZPF-3	250	125	>4000	>4000
Cipro	0.025	8	0.03	0.06

ZPF-1: Benzene; ZPF-2: ethyl acetate; ZPF-3: methanol; Cipro: Ciprofloxacin (reference drug).

correlates with the MIC for that particular bacterium/ antimicrobial combination. The present investigation confirms that there is a moderate degree of antibacterial activity against human pathogens as well particularly against Gram positive bacteria *(S aureus and MRSA)* in the ethyl acetate and methanol fraction of *Nodulisporium* sp.

REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST, A new generation of protein data base search programs, Nucl. Acids Res., 25: 3389-3402.

Fisher PJ, Petrini O (1990). A comparative study of fungal endophytes in xylem and bark of *Alnus species* in England and Switzerland. Mycol. Res., 94: 313-319.

Harrison L, Teplow D, Rinaldi M, Strobel GA (1991). Pseudomycins, a family of novel peptides from *Pseudomonas syringae,* possessing broad spectrum antifungal activity. J. Gen. Microbiol., 137: 2857-2865.

Liu CH, Zou WX, Lu H. Tan RX (2001). Antifungal activity of *Artemisia annua* endophyte cultures against phytopathogenic fungi, J. Biotechnol., 88: 277-82.

Munshi NA, Hassan DGH (2004). *Invitro* evaluation of some locally isolated microfungi for antagonism against mulberry Fusarial blight pathogen (*Fusarium pallidoroseum* (cook) SAAC.). J. Seriologia, 44(3): 341-346.

National Committee for Clinical Laboratory Standards. 1996. Methods for determining bactericidal activity of antimicrobial agents. Approved guideline M26-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.USA.

Strobel GA, Yang X, Sears J, Kramer R, Sidhu RS, Hess WM (1996). Taxol from *Pestalotiopsis microspore*, an endophytic fungus of *Taxus wallachiana*. J Microbiol., 142: 435-142.

Strobel GA, Long DM (1998). Endophytic microbes embody pharmaceutical potential. ASM News. 64: 263-268.

Wu TS, Leu YL, Hsu HC, Ou L F (1996). Nothpodytine A and B from *Nothapodytes foetida.* Phytochemistry, 42 (3): 907-908.

Wall ME, Wani MC (1977). Antineoplastic agents from plants. Ann. Rev. Pharmocol. Toxicol., 17: 117-132.