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Identification and production of *Azotobacter vinelandii* and its antifungal activity against *Fusarium oxysporum*

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Abstract

The phytopathogenic *Fusarium* species are one of the leading causes of loss in agricultural productivity. In search of an efficient bacterial antagonist, 19 soil isolates of *Azotobacter* sp. were screened for antagonistic activity against *Fusarium oxysporum* by agar well diffusion assay. The potential strain was identified as *Azotobacter vinelandii* by 16S rRNA sequencing. Optimum conditions for culturing *A. vinelandii* to obtain maximum antifungal activity were determined by varying temperature, pH, incubation period and NaCl and sucrose concentration. Maximum inhibition of *F. oxysporum* was observed at pH 7 and 8, 1% NaCl and 2% sucrose concentration and after 72 hr of incubation at 30°C temperature. *A. vinelandii* showed 44% higher yield of antifungal metabolite under optimized conditions. The minimum inhibitory concentration was 10 μg ml^{-1} for *F. oxysporum*. The FTIR analysis of purified metabolite showed presence of aldehyde, C-N, ester, aromatic ring, P-H stretch, and C-N stretch of alkyl amine in the structure. The purified antifungal metabolite of *A. vinelandii* showed effect on spore germination and mycelia morphology of *F. oxysporum*. The study revealed significance of *A. vinelandii* in controlling *F. oxysporum* and its promising application as a biocontrol agent in agriculture.

Key words

Azotobacter vinelandii, Antifungal metabolite, *Fusarium oxysporum*.

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Introduction

The genus *Fusarium* is considered as an important group of fungi due to its diversity, cosmopolitan nature and ability to cause plant diseases and storage rots (Summerell *et al.,* 2006). *Fusarium oxysporum* has received a considerable attention over the last few decades because of its ability to cause vascular diseases on a wide range of plants including non legumes, vegetable and fruits. The main target crop plants are tomato, cucumber, water melon, pepper, musk melon, bean, cotton and groundnut (Gordon and Martyn, 1997; Mayee, 2005 and Rasheed *et al.,* 2004). At present, effective means of controlling *F. oxysporum* includes use of chemical fungicides, crop rotation with non hosts of fungus or use of resistant cultivars (Agrios, 1988).

The increasing use of chemical fungicides is associated with several negative effects including environmental pollution, development of resistance and their non target environmental impacts. Furthermore, the growing cost of agrochemicals, particularly in developing world and consumer demand for pesticide free food has led to a search for substitutes of chemical fungicides. These concerns highlight the need for safe, potent and ecofriendly fungicide with higher degradability in nature. Microbial antagonists are widely used for the biocontrol of fungal plant diseases (Prapagdee *et al*., 2008; Yang *et al*., 2007).

Plant Growth Promoting Rhizobacteria (PGPR) are indigenous to soil and the plant rhizosphere and play a major role in the biocontrol of plant pathogens as they can suppress a broad range of bacterial, fungal, viral and nematode diseases. Hence, the use of biocontrol agent become a common practice in many regions of the world. A major group of rhizobacteria with potential for biological control of *Fusarium* sp is the *Pseudomonades*. Their multiple

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mechanisms in biocontrol include ability to produce wide variety of antibiotics, chitinolytic enzymes, siderophores and HCN (Banasco *et al.,* 1998; Kumar *et al.,* 2002). The major weakness of *Pseudomonades* as biocontrol agents is their inability to produce resistant structures like cysts or spores which complicates formulation of the bacteria for commercial use.

The free living, cyst forming aerobic nitrogen fixing organisms of the genus *Azotobacter* is one of the important PGPR that benefits plants in multiple ways. The beneficial effects of *Azotobacter* on growth and yield of various agriculturally important crop plants include their potential to fix N_2, produce vitamins and growth substances including indole acetic acid, gibberellins and cytokines (Verma *et al.,* 2001) which enhance root growth and aid in nutrient absorption and inhibit phyto-pathogenic fungi through antifungal substances and siderophores (Mali and Bodhankar, 2009).

The major issue in production of efficient biofertilizers using *Azotobacter* sp. is the search for efficient strains possessing an array of beneficial characteristics viz. a high rate of dinitrogen fixation, ability to produce growth promoting substances and broad spectrum antifungal activity towards phytopathogens (Kravchenko *et al.,* 2002). In search of efficient strain of *Azotobacter*, the present work was planned for isolation and screening for antifungal activity against *F. oxysporum* and production of antifungal metabolites.

Materials and Methods

Isolation, screening and identification of *Azotobacter* sp: The soil sample was collected from agricultural field of Nanded, Maharashtra, India. The soil sample was suspended in 10 ml autoclaved distilled water and serially diluted up to 10^{-8}. 0.1 ml of each dilution was spreaded on sterile Ashby's agar plates and the plates were incubated at 30˚C for 48 -72 hr. After incubation well isolated colonies were randomly selected on the basis of colony morphology and maintained on sterile Ashby's agar slants. Each isolate was characterized by gram staining, motility and cyst formation ability.

The *Azotobacter* isolates were grown at 30˚C on a rotary shaker (170 rpm) in 250 ml flasks with 100 ml production medium containing (g l^{-1}): Sucrose, 20; Yeast extract, 0.5; K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $FeCl_3$, 0.0016 and Na_2MoO_4, 0.001 for 72 hr. The bacterial cells were separated by centrifugation at 10,000 rpm for 30 min. Agar well diffusion method was used to check the presence of antifungal metabolites. The active culture of *Fusarium oxysporum* was diluted in sterile saline and the spore count was adjusted to 150 spores ml^{-1}. 0.1 ml of spore suspension was aseptically spreaded on potato dextrose agar plates. The 0.1 ml of cell free supernatant (CFS) was added in well. The experiment was carried in triplicate and the plates were incubated at 30˚C and observed for zone of inhibition around the well.

The efficient *Azotobacter* sp. isolate was identified on the basis of morphological and biochemical characters following Bergey's Manual of Systematic Bacteriology and 16 S r RNA sequencing analysis.

Effect of antifungal metabolite on fungal morphology: The selected strain of *Azotobacter* sp was subjected to different culture conditions. The factors used were temperature (20, 30, 40, 50 and 60˚C), pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), NaCl concentration (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%), sucrose concentration (1.0, 2.0, 3.0, 4.0 and 5.0%) and incubation period (24, 48, 72, 96, 120 and 144 hr).

The production media cultivated with efficient strain of *Azotobacter* sp was centrifuged at 10,000 rpm for 30 min to obtain CFS which was concentrated 20 fold by evaporation at 40˚C. The concentrate was extracted with double volume of ethyl acetate and chloroform. The metabolites were selectively precipitated with equal volume of methanol and the extract obtained was evaporated, dried, weighed and used for detection and purification by thin layer chromatography and column chromatography.

The methanol extract was spotted on TLC plates (silica gel G) and plates were developed using n butanol: acetic acid: water (4:3:2) as solvent. The separated components were detected in iodine chamber and its Rf values were determined.

The component of methanol extract was purified by using silica gel column chromatography. The elution was carried with n-butanol and chloroform graded concentration. The purified active fractions were analysed for functional groups by FTIR. The IR spectra were recorded on a FTIR spectrophotometer (Shimadzu FTIR Affinity 1, Japan) in the range of 400-4000 cm^{-1} (with KBr).

The activity of the finally purified antifungal metabolite was tested on PDA supplemented with a series of concentrations (0-50µg ml^{-1}) dissolved in same volume of methanol. The mycelial disks of *F. oxysporum* was placed on test plate and incubated at 28˚C. The lowest concentration that prevents visible mycelial growth was determined after determined after incubation for 4 to 5 days.

The effect of *A. vinelandii* antifungal metabolite on morphology of *F. oxysporum* was studied by observing the morphology of normal mycelium developed beyond the effective area of metabolites and the mycelium near the

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periphery of zone of inhibition with light microscope.

Results and Discussion

A total 19 isolates of *Azotobacter* sp were selected on the basis of their colony morphology. All isolates were Gram negative, motile, cyst formers and produced large (2 to 3 mm) mucoid, opaque and brown to yellow green colonies on Ashby's agar medium.

Of the 19 isolates, AZ7 isolate showed a maximum zone of inhibition (40mm) against *F. oxysporum* (Table 1). Many researchers also reported antifungal activity of *Azotobacter* sp. isolated from different soil samples. Cavaglieri *et al.* (2005) studied the effects of *Azotobacter* sp and *Arthrobacter* sp. on root colonization of *Fusarium verticillioides* and reported the growth inhibition and *in vitro* suppression of fumonisin B1 production by *Azotobacter armaniacus* RC2. Similarly, Mali and Bodhankar (2009) showed the inhibition of *Aspergillus terrus, Alternaria alternata, Aspergillus flavus* and *Fusarium oxysporum* by *Azotobacter chroococcum* isolated from rhizosphere soil of groundnut plants from Sangli district of Western Maharashtra. Agarwal and Singh (2002) also reported antifungal activity of *Azotobacter* sp. against *F. oxysporum, Rhizoctonia solani* and *Aspergillus* sp.

In the present study, AZ7 strain possessing highest antifungal activity was identified as *Azotobacter vinelandii*. The morphological and biochemical characters of the isolate are given in Table 2.

Table 1 : Antifungal activity of *Azotobacter* sp. isolates against *Fusarium oxysporum*

S.N.	Isolate	Diameter of zone of inhibition(mm)
1.	*AZ1*	12
2.	*AZ1*	-
3.	*AZ1*	15
4.	*AZ4*	29
5.	*AZ5*	18
6.	*AZ6*	20
7.	*AZ7*	40
8.	*AZ8*	-
9.	*AZ9*	30
10.	*AZ10*	-
11.	*AZ11*	-
12.	*AZ12*	15
13.	*AZ13*	18
14.	*AZ14*	18
15.	*AZ15*	-
16.	*AZ16*	15
17.	*AZ17*	-
18.	*AZ18*	-
19.	*AZ19*	-

Table 2 : Morphological and biochemical tests for identification of *Azotobacter* sp.

Test	Result
Grams nature	Negative, blunt rods
Motility	Motile
Cyst	Present
Pigmentation	Yellow green
Nitrate reduction	+
Amylase production	-
Utilization of carbon sources	
Glutamate	-
Rhamnose	-
Inositol	+
Oxaloacetic acid	+
D-Glucoronate	+
Sodium glycolate	-
Mannitol	-
Malonate	+
Benzoic acid	+
Galactose	-
Raffinose	+
Maltose	-
Glycerol	+

- = nil or absent

It has been reported that availability of nutritional requirements of organisms play an important role during metabolite synthesis process. Amongst various nutritional requirements, antifungal substance production has been known to be influenced by media components and cultural conditions, such as agitation, pH, temperature, carbon source and incubation time, which vary from organism to organism (Dahiya *et al.,* 2006). In the present study also cultural conditions were found to affect antifungal metabolite production by *A. vinelandii*. The parameters like incubation temperature, initial pH of the medium, salt concentration, sucrose concentration, and incubation period were optimized for maximum antifungal activity. The optimum conditions for antifungal metabolite production were pH 7-8, temperature 30°C, NaCl 1%, sucrose 2% and incubation period of 72hr. (Fig. 1 to 5).

Changes in external pH affect many cellular processes such as regulation of secondary metabolites biosynthesis (Datta and Kothary, 1993; Sole *et al.,* 1994). It was evident from the results that *A. vinelandii* (CFS) showed increased inhibition of *F. oxysporum* at pH 7.0 and 8.0 (27mm) after 72 hr of incubation. The *A. vinelandii* strain showed highest activity at 0.5 and 1% NaCl concentration after 96 hr of incubation. With increased salt concentration, the activity was found to be reduced whereas at 3% salt concentration 100% inhibition in the antifungal activity was observed.

Sucrose is an excellent source of carbon for bacterial growth and may involve in synthesis of many secondary metabolites (Espeso *et al.,* 1993). Hence, the effect of sucrose

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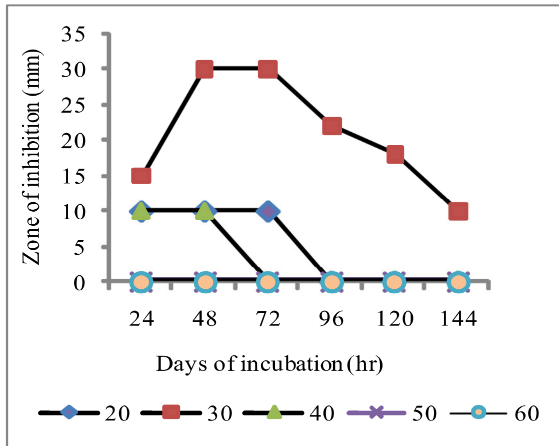

Fig. 1: Effect of temperature on antifungal activity of *A. vinelandii*

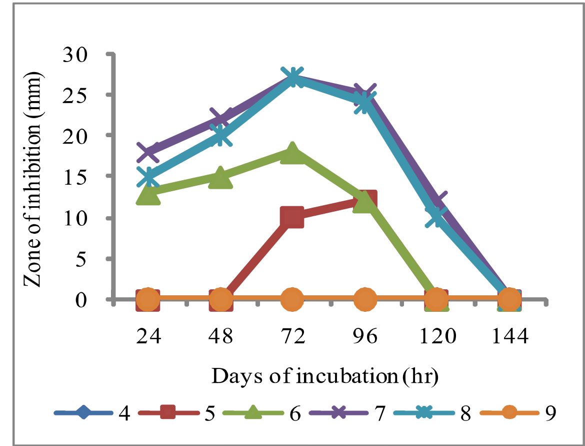

Fig. 2: Effect of pH on antifungal activity of *A. vinelandii*

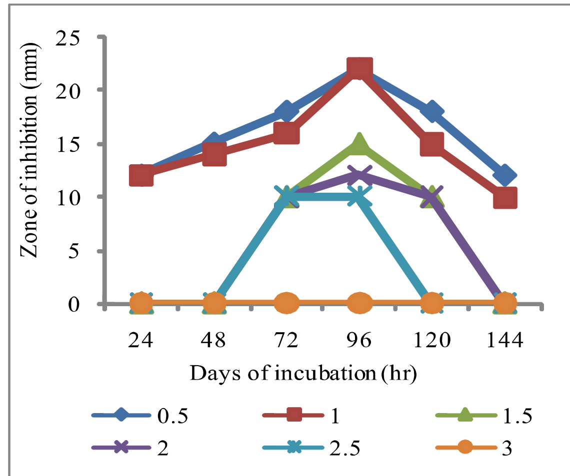

Fig. 3: Effect of salt concentration on antifungal activity of *A. vinelandii*

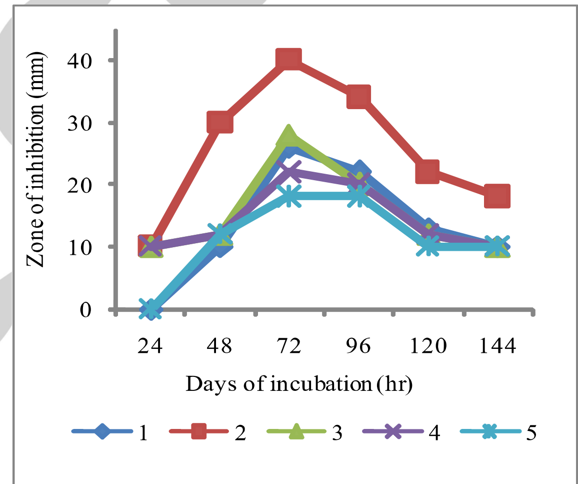

Fig. 4: Effect of sucrose concentration on antifungal activity of *A. vinelandii*

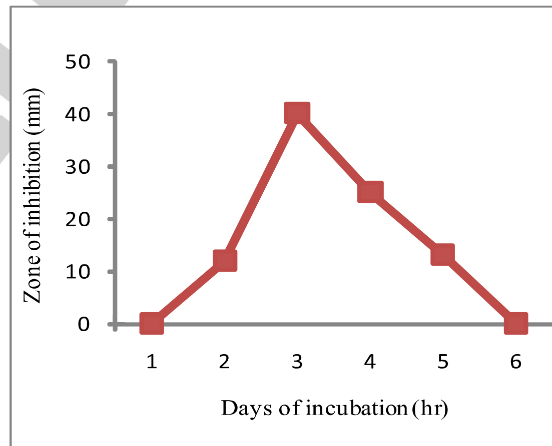

Fig. 5: Effect of incubation period on antifungal activity of *A. vinelandii*

concentration on the production of antifungal compound was studied. Maximum zone of inhibition (40mm) was produced by *A. vinelandii* at 2% sucrose concentration after 72 hr indicating it is the most suitable concentration for antifungal metabolite production.

The antifungal metabolite produced by *A. vinelandii* was analyzed by TLC, column chromatography and FTIR. The single spot observed by TLC showed Rf value of 0.53. The spectra of purified compound recorded on FTIR spectrophotometer showed the major peaks at 2700.34, 2331.94, 1734.01, 1541.12, 1288.45 and 1039.63 indicating presence of aldehyde, C-N, ester, aromatic ring, P-H stretch, and C-N stretch of alkyl amine as prime functional groups in the metabolite (Table 3). Chetverikov and Longinov (2009) investigated the antifungal metabolites produced by *Azotobacter vinelandii* by HPLC, NMR, IR and elemental analysis and demonstrated the presence of sucrose tetraamine polyphosphates in the metabolite exhibiting antifungal activity. The bands in IR spectra indicated the presence of phosphate, amine, C-H and O-H stretch in the structure. However, the presence of aromatics, ester, aldehyde and C-N group indicated presence of possibly some novel functional groups in the structure that needs to be characterized further.

The antifungal activity of the metabolite was examined on PDA containing its varying concentrations. The MIC of the metabolite was 10μg ml^{-1} for *F. oxysporum*. Reports are available indicating different values of MIC for antifungal metabolites extracted from diverse organisms. Lee *et al.* (2003) observed the effect of aerugine from *P. fluorescens* strain MM-B16 on phytopathogenic fungi including *F. oxysporum* and showed the insensitivity of *F. oxysporum* to 100μg ml^{-1} concentration of aerugine. Present study showed the significance of *A. vinelandii* antifungal metabolite in exerting its effect even at 10μg ml^{-1} concentration. However, antifungal activity of antibiotic KB-8A from *Bacillus polymyxa* was shown to be effective at lower concentrations of 12.8μg ml^{-1} for *F. oxysporum* (Hyun *et al.,* 1999). The effect of antifungal metabolite on fungal cell morphology was also studied in *F. oxysporum* as a test organism. Significant inhibition of the mycelium development compared to the control was observed and the fungal hyphae were characterized by distinct changes in morphology. In the presence of metabolite, segmented and branched mycelium was observed. When the fungal mycelium from affected zone was grown on potato dextrose agar medium, the growth was found to be delayed markedly as compared to control. A similar effect was observed between *Fusarium* and antagonistic bacilli by Melent'ev and Galimzyanova (1999).

Table 3: FTIR analysis of *A. vinelandii* antifungal compound

Wave no. in IR spectra	Functional group
2700.34	Aldehyde stretch
2331.94	C-N stretch
1734.01	C=O of Ester
1541.12	Aromatic ring
1288.45	P-H stretch
1089.63	C-N stretch of alkyl amine

Thus, the promising antifungal activity exhibiting strain of *A. vinelandii* was isolated, identified and optimized for maximum antifungal activity. The effect on fungal morphology demonstrated that the antifungal metabolite interferes with the developmental stages of fungi. Therefore, the *A. vinelandii* strain used in study may be used as a promising bio-control agent against phytopathogenic *F. oxysporum*.

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