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Isolation of antagonistic actinomycetes species from rhizosphere of non bt-cotton

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Abstract
The plant microbe interaction in the rhizosphere is one of the major factors regulating the health and growth of plants. Actinomycetes are common filamentous soil microorganisms important in maintaining a satisfactory biological balance in the soil, largely because of the ability to produce antibiotics. In the present study Antagonistic Actinomycetes species was isolated from rhizosphere of Non Bt cotton. Eight types of actinomycetes colonies were isolated from crowded plate method and were screened primarily by Giant colony technique. Three strains with best antifungal activity were further screened by Well Diffusion method and the best member which has good antifungal activity was selected and named as NBtAS. This strain was studied for it's morphological, physiological characteristics according to Bergey's Manual and further studied by molecular characterization and was identified as *Streptomyces violatus.* The antagonistic nature of the isolated strain was determined for its anti-fungal activity by Well Diffusion method, MIC and Inhibition of phytopathogenic fungi like *A. alternata, F. moniliformae, M. phaseolina, R. solani*and *A. niger*in liquid medium. The results indicate that *Streptomyces violatus*isolated from rhizosphere of Bt cotton has Good Antifungal activity and it was more effective against *Macrophomenaphaseolina*when compared with other test fungi.

Keywords: Rhizosphere, Antagonistic, Crowded plate, Giant colony technique, Well Diffusion method, phytopathogenic, MIC.

1. Introduction
Cotton (*Gossypiumherbaciumarboreu*) was the one of the important commercial crop in India. The Rhizosphere contains a large and majority of the soil biota. The plant microbe interaction in the rhizosphere is one of the major factors regulating the health and growth of plants. Soil bacteria living in the rhizosphere can enhance plant growth by several mechanisms like antagonism against plant pathogens, solubilization of phosphates [8], production of phytohormones [2], siderophores [10], antibiotic production [16] inhibition of plant ethylene synthesis [9] and induction of plant systemic resistance to pathogens [11]. The study of rhizosphere is important as far as control of soil pathogens which pass through the rhizosphere and infect root system.

Biological control is a common phenomenon in a soil ecosystem. It is a site for complex diverse microbe mediated processes. Several microorganisms like Actinomycetes secrete low levels of antibiotic compounds as their secondary metabolites. Many of them are effective against bacteria, fungi and actinomycetes which maintain natural soil health. This is a continuous process which can inhibit or kill some of the plant pathogens in that vicinity. Actinomycetes are common filamentous soil microorganisms important in maintaining a satisfactory biological balance in the soil, largely because of the ability to produce antibiotics. They are also known to be actively involved in degradation of complex organic materials in soils and contribute to the biogeochemical transformations. Most of the actinomycetes are capable of producing wide variety of cell wall degrading enzymes like chitinases, glucanases, cellulases, hemicellulases, amylases etc. These are also known to produce several antifungal compounds that are being exploited commercially for the control of several microbial plant diseases.

Materials and Methods
Soil sampling
The study area covers Khammam district, Telangana State, India. The rhizospheric soil samples were collected by shaking the roots vigorously to separate the loosely bound bulk soil. The soil samples at pre-vegetation and post-harvest stage were collected from 0-15 cm

depth using a 5 cm diameter soil corer Amith Kishore Singh *et al.,* 2013[1]. After removal of plant debris, the soil samples were sieved using 2mm mesh size sieve and air dried. Then they were labeled and transported to the laboratory in polyethylene bags and stored at 4^0C, and were further used for the isolation of antagonistic Actinomycetes.

Isolation of actinomycetes by Crowed plate method

The rhizosphericsoil (1gm) was suspended in 10 ml of sterile 0.85% NaCl solution, serially diluted (10^{-1} to 10^{-6}), centrifuged at 500 rpm for 20 minute to disperse the spore chains. The suspension was allowed to settle for 1hr and plated on to Starch Casein Agar (SCA) [14]. The plates were incubated at 28±2^{0}C for 84 hrs. The plates were observed intermittently during incubation for whitish pin point colonies with a zone of inhibition around them. The pin point colonies with inhibitory zone were selected and purified by multiple streaking methods. The isolated eight types of actinomycetes colonies from Non Bt rhizosphere were maintained on SCA slants at 4 ^{0}C [13].

Primary screening by Giant colony technique

Single streak of each Actinomycete was made on Modified nutrient agar (glucose 5gm, peptone 5gm, beef extract 3gm, NaCl 5gm, agar 15 gm at pH 7) and incubated at 28±2ºC for 4 days to test antibacterial activity. After observing a ribbon like growth of the Actinomycetes, the pathogenic bacteriae (*Escherichia coli, Klebsiellapneumoniae, Staphylococcus aureus, Proteus vulgaris, Pseudomonaspyogenes*) were streaked at right angles to the original streak of each actinomycete and incubated at 37ºC. The inhibition zone was measured after 24 h [12]. FiveFungal cultures of agriculture importance (*Alternariaalternata, Fusariummoniliformae, Macrophomenaphaseolina, Rhizoctoniasoloni*and*Aspergillusniger*) were used to determine the antifungal activity of the isolated actinomycetes strain. To test the antifungal activity single streak of actinomycetes were made on Kuster's agar [14] and the test fungal pathogens were streaked at right angles to the original streak of each Actinomycete and incubated at 28±2ºC. The inhibition zone was measured after 7days of incubation [5].

Secondary screening of selected strains by Well Diffusion method

Five isolates which shown most effect on phytopathogenic fungi, were selected for secondary screening. It was carried out by Well Diffusion method.

Preparation of fermentation broth

The strains were cultured on Starch Casein Agar slants at 28±2^{0}C for 2 weeks for sporulation. The mature spores were inoculated in Starch Casein Broth. The fermentation set up was incubated on rotary shaker at 200 rpm for 10 days at 28±2^{0}C. The fermented broth was centrifuged at 10,000 rpm at 4^0C for 20 min. The supernatant was filtered using 0.2 µm filters and the filtrate was collected as antibiotic sample [4].

Testing of antibiotic sample from antagonistic Actinomycetes

To determine the antagonistic activity the phytopathogenic fungi were cultured in AsthanaHakuer's broth [5] at 28^0C for 5 days. The cultures were swapped on Potato Dextrose Agar (PDA). Four wells (6 mm) were prepared in each seeded agar plate and each well was filled with 100 µl of the fermentation broth of the selected strains. These PDA plates were incubated at 28±2^{0}C for 5 days. After the incubation the diameter of the inhibition zone was measured. Depending on the zone of inhibition, one strain was selected and named as NBtAS.

Characterization for the taxonomic position of the selected Actinomycetes strain NBtAS

The taxonomic position of the selected strains was determined by studying their morphology, fine structure and spore chain morphology by Gram staining and SEM , Colonial Characteristics were observed for aerial mass color, melanin production, diffusible pigments, reverse side pigmentation of the colony.Nutrition and growth characteristics were determined by growth on different media like Glucose Yeast Extract Malt extract Agar (GYEA) Oat meal Agar (OA) Glycerol Asparagine Agar (GASp) Peptone Yeast extract Iron Agar (PYIA) Tyrosine Agar (TA), Nutrient agar (NA), Malt Yeast Extract Agar (MYEA) and Starch Casein Agar (SCA) [19]. Utilization of Carbon and Nitrogen Sources, Antibiosis and resistance to antibiotics was studied.Physiological characterization was also determined by studying Growth at different temperatures, pH, and Salt concentration. Enzyme activity was studied by testing Chitinolytic activity, Lipolysis activity, lecithinase activity, pectin hydrolysis, urease hydrolysis, starch hydrolysis and gelatin hydrolysis, Denitrification Test, nitrate reduction test and H_2S production test [19, 18].

Determinaton of antagonistic activity by Well diffusion method

The plates were seeded with test fungal inoculum (0.1ml) and wells were punctured (8 mm in diameter) with sterile cork borer. The wells were filled with filtrate of the culture suspension in various concentrations i.e 25 µl/ well, 50 µl/ well, 75 µl/ well and 100 µl/ well. A well with a standard antibiotic (Nystatin 100 µl/well) was also set for reference. The entire set up was incubated at 28±2 ^{0}C for 4 days. Clear inhibition zone around wells was measured in millimeters [15].

Calculation of Activity Index

Activity index of NBtAS was calculated by comparing the inhibition area of the test sample with that of standard antibiotic [17].

Activity Index =Zone of inhibition in mm of test sample ÷ Zone of inhibition in mm of standard antibiotic.

Inhibition of fungal pathogens in Czapeck's Broth

The potential antagonistic activity of the NBtAS culture was tested against test fungal pathogens in Czapeck's Broth (CZB). Fungal inoculum of 0.1 ml capacity was inoculated in to 50 ml of CZB to which 0.5 ml of culture filtrate of NBtAS was inoculated separately and incubated for 4 days at 28±2^{0}C. All the experimental set up was carried out in triplicates.

The difference in dry weight between the mycelia grown with and without NBtAS culture were measured [20].Cultures were passed through pre weighed Whatman No 1 filter paper and dried overnight in an oven at 60^0C

and reweighed. Dry weights of fungal cultures were calculated and compared.

Measurement of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory concentration (MIC) is the least concentration of the antimicrobial agent in µg/ml that will inhibit growth of the phytopathogenic fungi. MIC value of NBtAS was determined by serial two fold dilution in Sabouraud Dextrose Broth (SDB) with the dilution ranging from 20-120 µg(20, 40, 60, 80,1 00, 120 µg/ml). The100 µl of each dilution was tested against phytopathogenic fungi by well diffusion assay. The definite zone of inhibition of any dimension surrounding the well was measured accurately to the nearest millimeter by means of ruler [7, 3, and 6]. Depending upon the inhibition zone the minimum concentration at which the fungal pathogens were inhibited was noted.

Results and Discussion

The representative soil sample for the isolation of antagonistic Actinomycetes from Bt and NBt fields was the rhizospheric sample collected at 60 Days growth stage.

The antagonistic actinomycetes were isolated by crowded plate method by maintaining triplicates of SCA (Starch Casein Agar) plates for Bt soil samples. Similar method was followed for NBt soils also. Whitish pin point colonies with the zone of inhibition were observed in a good number on SCA plate with 10^{-5} dilution. Eight from NBt sample, were selected. All the selected cultures were sub cultured to get pure cultures. All the colonies from NBt soil sample were named as NBtASI, NBtASII, NBtASIII, NBtASIV, NBtASV, NBtASVII and NBtAS VIII. Further, all these colonies isolated were screened for their antagonistic activity against test phytopathogenic fungi and pathogenic bacteria.

The eight isolates from NBt rhizosphere were tested for antagonistic activity against phytopathogenic fungi, and pathogenic bacteria by giant colony technique and zone of inhibition was compared among the isolates. The order of the isolates for their antagonistic activity was NBtASII, NBtASI, NBtASIII, NBtASVII, NBtASIV, NBtASV, NBtASVI, and NBtASVIII (Table 1). The three isolates (NBtASII, NBtASIII, NBtASI) with better activity were selected for further screening to select one strain.

Table 1: Zone of inhibition against Fungi and Bacteria on Giant colony Technique by NBt isolates

Test fungi	Zone of inhibition (mm)							
	NBtAS I	**NBtASII**	**NBtAS III**	**NBtASIV**	**NBtASV**	**NBtAS VI**	**NBt AS VII**	**NBt AS VIII**
A. alternata	3	3	5	2	3	2	3	2
F. moniliformae	4	4	2	3	2	1	2	1
M. phaseolina	4	6	3	2	1	1	2	2
R. solani	5	8	4	2	3	4	4	3
A. niger	3	6	2	1	3	2	1	2
TestBacteria								
E .coli	4	6	3	3	4	3	3	3
K .pneumoniae	5	5	3	4	3	2	2	2
S. aureus	3	7	2	3	2	2	3	3
P. pyogens	6	9	4	3	3	3	3	4
p. valgaris	4	6	2	2	3	3	2	3

Secondary screening of selected strains by Well Diffusion method

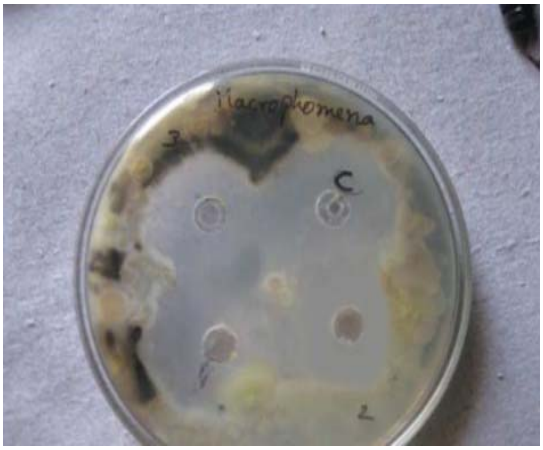
Three selected isolates from Non Btrhizospheric soils were further screened for their antagonistic activity against test fungi by Well Diffusion method. The zone of inhibition for each of the isolates was measured and compared to get one best isolate from NBt environment (Table 2)

NBtASII was found to have strong inhibition against *M.Phaseolina*and moderate inhibition against *A. alternata*, *F. moniliformae, R. solani, A. niger*, whereas NBtASI and NBtASIII have lesser activity comparatively (Photo 1 and 2).

Table 2: Antifungal activity of the isolates from NBt soils by Well Diffusion method

Test fungi	Zone of inhibition (mm)		
	NBtAS 1	**NBtAS II**	**NBtAS III**
A. alternata	+	++	++
F. moniliformae	++	++	+
M. phaseolina	+	+++	+
R. solani	++	++	++
A. niger	+	++	+

Weak inhibition 5-9mm(+), moderate inhibition10-19mm(++),strong inhibition>20mm(+++).



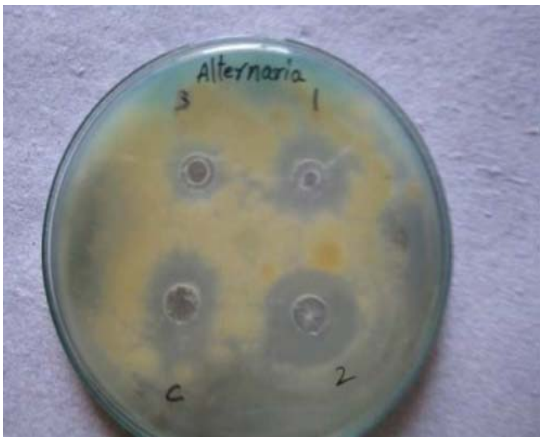


Photo 1 and 2: Zone of inhibition against *M. phaseolina* and *A. alternata*byNBtASII (1), NBtAS I (2), NBtASIII (3) and control (C)

Depending on the results obtained from primary and secondary screening NBtASII from NBt environment were found to have good antagonistic property. They were named as NBtAS for further study.

Characterization for the taxonomic position of the selected actinomycetes strain NBtAS

The taxonomic position of the selected strain was determined by studying their morphology and fine structure, colonial characteristics, nutrition and growth, finally genetics according to the guidelines of *Bergey's Manual of Systemic Bacteriology*Vol IV [19] and *International Streptomyces Project I* [18].

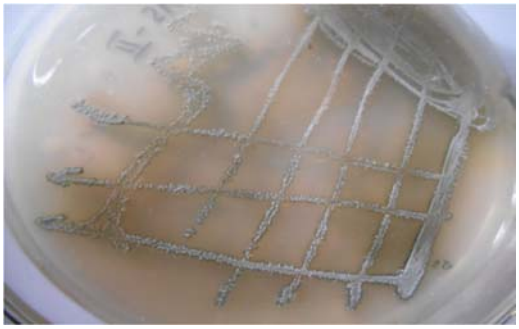
Growth characteristics of BtAS was observed using different types of media such as ISP2 (Malt- Yeast Extract Agar), ISP5 (Glycerol Yeast extract Malt extract Agar), ISP6 (Peptone Yeast extract Iron Agar), SCA (Starch Casein Agar), and Nutrient Agar (NA). All the characteristic features on different media were observed for different growth patterns and recorded.

Growth characteristics of NBtAS were observed using different types of media such as ISP2 (Malt Yeast Extract Agar), ISP5 (Glycerol Yeast extract Malt extract Agar) ISP6 (Peptone Yeast extract Iron agar), Starch Casein Agar (SCA), and Nutrient Agar. All the characteristic features on different media were recorded. Good growth on all the media was found with various colors. Diffusible pigments were observed on all the media except on NA. Melanin production was observed on SCA and PYIA. The observed morphological characteristic were recorded (Table.3).

Table 3: Morphology and fine structure of NBtAS

Characteristics	NBtAS
Spore mass	Dark grey
Spore chain morphology	Short length spore chain
Spore surface	Rough and spiny
Reverse side pigment	Dark brown to black
Diffusible pigments	+
Melanin	+
Spore size	745-755nm
Spore shape	Rectangular to rod shape

(+) Positive; (-) negative.



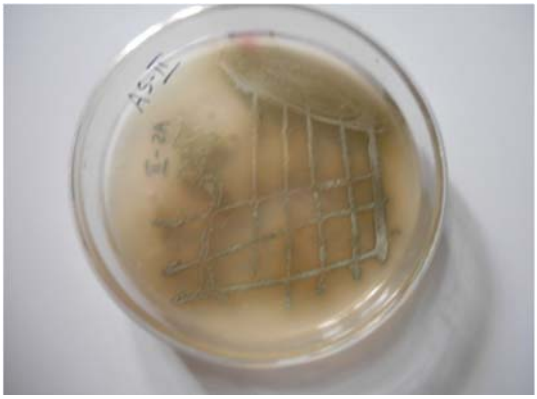


Photo3: Colony morphology of NBtAS

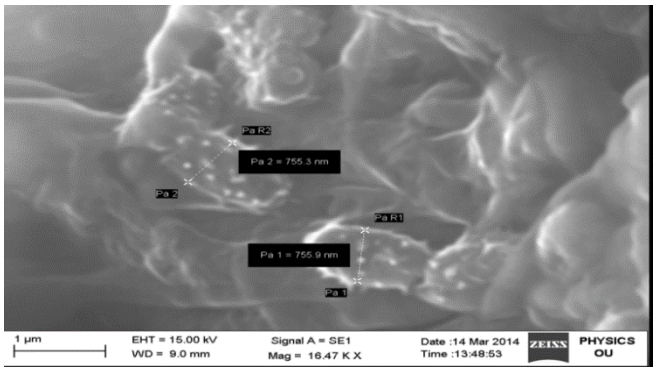


Photo4: SEM Image showing NBtAS spore Diameter (755 nm)

Physiological characteristics

A temperature range of 23-40^{0}C was found suitable for the growth ofNBtAS while 28^{0}C was found to be optimum and showed temperature tolerance up to 45^{0}C. The strain was grown in SCA at pH value 4.5-8.5 for 10 days and found that pH 6.5 was optimum for growth. Growth was observed in the range of 1-9% NaCl concentrations and 3% NaCl was found optimum for growth. The strain was incubated for 15 days with good aeration and 3% NaCl at pH 6.5 and at 28^{0}C. The growth was measured on alternate days and maximum mycelial dry weight was observed at 7 days of incubation (Table 4).

Table 4: Physiological characteristics of NBtAS

Physiological characteristics	NBtAS
5^0C	--
15^0C	--
Temperature range28^0C-45^0C	+
Maximum temperature tolerance	45 ^{0}C
Optimum temperature	28^0C
pH range	6-8
Optimum pH	6.5
NaCl range	1-9%
Optimum NaCl	3%
Incubation period range	5-15days
Optimum incubation period	7 days

(+) positive (-) Negative

Degradation and enzyme activity studies

In case of NBtAS, tests for catalase, oxidase, lipase, phosphatase and denitrification and H_2S production were positive. Test for Nitrate utilization and lecithinase were negative. Degradation of casein, cellulose, pectin, starch, chitin and urea was positive (Table 5).

Table 5: Degradations and Enzyme activity studies of NBtAS

Degradation/hydrolysis:	NBtAS
Casein	+
Cellulose	+
Pectin	+
Starch	+
Chitin	+
Urea	+
Catalase	+
Oxidase	+
Lipase activity	+
Denitrification	+
Nitrate reduction	--
Lecithinase activity	--
Phosphatase	+
Hydrogen sulphate test	+

(+) positive (-) Negative.

Nutrition and growth

Carbon, Nitrogen utilization pattern and other factors effecting were studied according to standard methods described for Actinomycetes.

The NBtAS strain used D-Sucrose, L-Raffinose, L-Rhamnose, D-Fructose, D-Glucose, D-Mannitol and D-Xylose. Acid was not formed by these carbon sources. L-Asparagine, L-Phenylalanine, L-Histidine were utilized but L-Hydroproline cannot be utilized by NBtAS (Table 6).

Table 6: Utilization of carbon and nitrogen sources by NBtAS

Utilization of carbon sources	NBtAS
D-Sucrose	+
L-Raffinose	+
D-Mannitol	+
L-Rhamnose	+
D-Fructose	+
D-Glucose	+
D-Xylose	+
L-Aspergine	+
L-Phenylalanine	+
L-Histidine	+
L-Arginine	+
L- Hydroproline	--

(+) Positive, (-) Negative.

Antagonistic property and Resistance to antibiotics exhibited by NBtAS

NBtAS exhibited antagonism towards *Escherichia coli*, *Klebsiellapneumoniae*, *Staphylococcus aureus*, *Pseudomonas pyogenes*and*Proteus vulgaris*, and NBtAS was resistant to Rifampicin (50µg/ml) and Penicillin-G (50µg/ml) and sensitive to Oleandomycin (50µg/ml) and Neomycin (50µg/ml) (Table 7).

Table 7: Antagonistic property and resistance to antibiotics exhibited by NBtA

Antagonistic to	NBtAS
E. coli	+
S. aureus	+
P. pyogenes	+
K. pneumoniae	+
p. vulgaris	+
Resistance to	
Rifampicin (50µg/ml)	+
Penicillin G (50µg/ml)	+
Oleandomycin(50µg/ml)	_
Neomycin (50µg/ml)	_

(+) positive, (-) negative.

From the above colonial, morphological, physiological and nutritional, enzymatic degradation studies, NBtAS was identified as member of genus *Streptomyces*, *Category I*, cluster *Streptomyces cyaneus* and the strain has 77.5% similarity level with *Streptomyces violatus.*

The taxonomic status of NBtAS was *Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.*

Determination of the antibiotic activity for the fermentation product of NBtAS

The representative filtrate for *S. violatus* was prepared by fermentation setup in which pre inoculated SCB with *S. violatus* was maintained with good aeration and was incubated at 28 ± 2^0C for 10 days at neutral pH. After incubation the content of the flasks was filtered through Whatman No.1filter paper and filtrate was collected into a vessel.

Determinaton of antagonistic activity by Well Diffusion method

Antifungal activities of *S. violatus* were tested against selected phytopathogenic fungi by Well Diffusion method. The diameter of Zone of Inhibition was tabulated. Optimum antifungal activity for *S. violatus* was observed against *M. phaseolina* (Photo 5), *R. solani*, *F. moniliformae*, *A. alternata*and A. *niger*. Culture filtrates of *S. violatus* at concentrations of 25µl showed activity against *M. phaseolina*, *R. solani* and at 50 µl/ ml the activity was observed against all the test fungi (Table 8). The measured zone of inhibition was near to standard antibiotic with concentration of 100µl/ ml. Concentrations of 75 and 100 µl/ml was found to be higher than standard i.e. nystatin 100 µl/ ml antibiotic.

Photo 5: Zone of inhibition against *M. phaseolina* by *S. violates*

Table 8: Zone of inhibition by *S. violatus* by Well Diffusion method

Test fungi	Concentration of culture filtrate(µl/ml)				
	25	50	75	100	Nystatin(100)
	Zone of inhibition (mm)				
A.alternata	7	11	14	21	11
F.moniliformae	8	12	13	17	15
M. phaseolina	9	10	13	24	12
R. solani	12	15	17	23	18
A.niger	6	9	12	19	10

In case of *S. violatus*high activity index was observed against *M. phaseolina*followed by *F. moniliformae*, *A. alternata*, *R. solani*and*A. niger*. Depending on activity index the test plantpathogenic fungi for *S. violatus*was *M. phaseolina*. The future studies were conducted with *M. phaseolina* against *S. violatus*.

Inhibition of fungal pathogens in Czapeck's Broth:

The selected pathogenic fungal cultures i.e. *M. phaseolina* for *S. violates* were inoculated into Czapeck's broth and incubated. After incubation a reduction in dry weight of test fungi was observed. There was more than 55% reduction in dry weight of test fungi from 4.6 to1.9 mg /50 ml. Substantial reductions in dry weights of fungi by these strains was due to their strong antagonistic nature exhibited by the isolate from NBt rhizosphere.

Measurement of Minimum Inhibitory Concentration (MIC) for *S. violatus*

MIC values were determined by broth dilution procedure using two fold dilutions of antibiotic substance in Sabouraud Dextrose Broth (SDB) with the dilution ranging from 20-120 µg/ml (20, 40, 60, 80, 100 and 120 µg/ ml) [3 and 20]. Each dilution of 50 µl was tested against phytopathogenic fungi by Well Diffusion assay. Depending upon the zone of inhibition the minimum concentration at which the fungal pathogens were inhibited was noted for *S. violatus*. MIC values were in the range of 20-40 µg/ml for *S. violatus* against all the test pathogenic fungi. *M. phaseolina*was inhibited at 20µg/ml concentration of the antibiotic substance from *S. violatus.*

Conclusions

In the present study antagonistic Actinomycetes was isolated from the rhizosphere of Non Bt cotton in the field conditions. The isolated strain was characterized and determined its antifungal activity by using basic techniques. This isolated strain *S. violatus*has good antifungal activity against all the test fungi and has shown highest activity against *M. phaseolina* which was a common fungal plant pathogen in the rhizosphere and causative agent of several root rots. Determination of optimum conditions for the fermentation product, it's other applications, molecular characterization of antibiotic substance was the scope of this study.

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