

***In vitro* Antagonistic Activity of Some Actinomycetes and Fungal spp. Isolated from Rhizosphere of Maize against *Fusarium* sp. by Bio-control Dual Inoculation Method**

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Received: 11.11.2018 | Revised: 21.12.2018 | Accepted: 28.12.2018

ABSTRACT

Rhizosphere actinomycetes are promising biocontrol agents for use in agriculture and have been isolated from various plant species. In the present investigation, actinomycetes were isolated from rhizospheric soil of maize to test antagonistic activity against Fusarium verticillioides and fungal strain (T. viride) were procured from National fungal culture collection of India (NFCCI), T. harzianum and A. niger were isolated from soil to test their antagonistic effects against Fusarium verticillioides. In the present study bio-control dual inoculum method were followed to evaluate the bacterial and fungal strains against the pathogen and reveals that Single Bacterial Inoculum (SBI) of S. cinereus shows more effective in controlling the growth of Fusarium verticillioides (28 mm in treated plate) compare to control (45 mm fungal growth). But in Dual Bacterial Inoculum (DBI), the combination of S. albosporous and S. cinereus shows more effective (08 mm fungal growth in treated plate) compare to control (52 mm fungal growth). Similarly, in Single Fungal Inoculum (SFI), T. harzianum were more effective (18.8 mm fungal growth) and in Dual Fungal Inoculum (DFI), T. viridae and T. harzianum act as a more effective (2.5 mm fungal growth) against Fusarium verticillioides compare to controls (60 mm and 52 mm fungal growth respectively). Overall interpretation of the present work reveals that DBI and DFI were more effective than SBI and SFI against Fusarium verticillioides.

Key words: Antagonistic, Actinomycetes, Phytopathogenic fungi, Rhizosphere and Biocontrol dual inoculation.

INTRODUCTION

There have been many recent studies on the use of microbial antagonists to control diseases incited by soil-borne plant pathogenic bacteria and fungi. Actinomycetes are known as

producers of antibiotics and other biologically active substances with high commercial value (enzymes, enzyme inhibitors, plant growth factors, etc.)²¹.

Cite this article: Mahadevaprasad, G. and Swamy, V.A.L., *In vitro* Antagonistic Activity of Some Actinomycetes and Fungal spp. Isolated from Rhizosphere of Maize against *Fusarium* sp. by Bio-control Dual Inoculation Method, *Int. J. Pure App. Biosci.* 6(6): 1225-1234 (2018). doi: <http://dx.doi.org/10.18782/2320-7051.7239>

Evidences indicated that actinomycetes are important in the rhizosphere because they can influence plant growth and protect plant roots against invasion by root pathogenic fungi^{3,20}. Several workers isolate actinomycetes from rhizosphere region of maize^{13,5,4}. *In vitro* and *in vivo* antagonistic activities of rhizosphere actinomycetes against plant pathogens have been reported^{2,18,19}.

In agriculture, plant growth-promoting and biocontrol microorganisms have emerged as safe alternatives to chemical pesticides. *Streptomyces* spp. and their metabolites may have great potential as excellent agents for controlling various fungal and bacterial phytopathogens²³.

Fungal based biological control envoys have gained wide recognition next to bacteria⁹. In this context *Trichoderma* spp. was the cynosure of the many researchers who have been contributing to biological control pursuit through use of fungi^{6,11,17}. In dual cultures of actinomycetes, the strains were assayed for antagonistic activity against various fungal pathogens, *Verticillium dahliae*, *Alternaria solani*, *Fusarium solani* and *Geotrichum candidum*⁸. The present study was undertaken with a view to test the potential of actinomycetes and *Trichoderma* spp. as biocontrol agents against a phytopathogenic fungus, *Fusarium verticillioides*.

MATERIAL AND METHODS

1.1. Isolation of Bacteria:

The antagonistic bacterial strains were isolated from native soil by serial dilution, maintained on starch – casein agar medium (SCA), Yeast extract-malt extract agar medium, King's medium (KMB) and Ashby's Mannitol Agar (AMA) and bacterial culture used both as pure culture as well as formulation.

About 4 g of each of the above soil samples was suspended in 25 ml of sterile water in 100 ml conical flask and stirred for half an hour on a rotary shaker. The suspension was serially diluted, 1 ml of each of these dilutions was added to 50 ml of each of the above sterile molten agar medium maintained at 40- 45 °C thoroughly mixed and

poured into sterile Petri plates (6" dia). Nystatin, (25µg/ml) and rifampicin (5µg/ml) were added to the media to suppress the growth of fungi and bacteria respectively. Antifungal and antibacterial antibiotics were sterilized by filtration and added aseptically to the sterile medium before plating. All the plates were incubated at 28° C for 7 to 14 days. After 7 days, the selected actinomycetes colonies were isolated from different plates and transferred to starch-casein agar slants. The slants were incubated at 28°C for 7 to 10 days. A total of five actinomycetes were isolated from the above samples. The isolates were pooled together and cultures which appeared identical to the naked eye were eliminated using the following criteria: Colour of the aerial mycelium, reverse colour and soluble pigment. The antimicrobial spectrum of these five isolates was determined by subjecting the organisms to submerged fermentation and assaying the broth for antimicrobial activity by cup plate assay.

1.2. Fungal cultures:

Fungal strain, *T. viride* (ATCC9275) were procured from National fungal culture collection of India (NFCCI), Agharkar Research Institute (ARI), Pune, India. *T. harzianum* and *A. niger* were isolated from soil to test their antagonistic effects against *Fusarium verticillioides*. Distinct bioagents were isolated from the Rhizosphere of maize and other parts of the host plant and maintained on PDA medium. Isolated bio agents were mass multiply on corn grain/nutrient broth medium

1.3. In vitro antagonistic bioassay

Modified Agar overlay method was employed for the screening of different rhizospheric bacteria to inhibit the growth of *F. verticillioides*. Isolates were screened for antifungal activity in a dual culture assay on SC agar plates. Isolates were grown in two lines on the plate under normal conditions. After incubation, the plate was overlaid with malt extract soft agar containing fungal spores. If clear zones of inhibition were present on the plates, isolates were considered to possess antifungal activity and selected for further

evaluation. The bacterial isolates was evaluated for their activity towards pathogenic fungi by dual- culture *in vitro* assay as followed by Gangwar *et al.*⁷ and direct dual culture method¹⁰. Pathogenic fungal discs (8 mm in diameter) of 5 days old were placed on PDA at 28 °C at the center of plates. Two actinomycete discs (8 mm) 5 days old, grown on yeast malt extract (YM) incubated at 28 °C were placed on opposite sides of the plates, 3 cm away from fungal disc. Plates without the actinomycete disc serve as controls. All the plates were incubated at 28 °C for 14 days and colony growth inhibition (%) was calculated by using the formula:

$$C - T/C \times 100,$$

Where C is the colony growth of pathogen in control and T is the colony growth of pathogen in dual culture. The zone of inhibition was measured between the pathogen and actinomycete isolates.

Other method for calculation of inhibition by fungal inoculums includes, each of the fungal isolates was point inoculated at sides 3cm from the center of the plate and after that six day old culture of *F. verticillioides* was point inoculated in the center of the plate. Control plate was only point inoculated with *F. verticillioides*. The plates were sealed with parafilm and incubated at 28±2°C for 4-5 days. Antagonistic activity was investigated for four to seven days after incubation at room temperature (28±2°C). After 5 days of incubations the inhibition in growth of pathogen was calculated by the formula:

$$\text{Inhibition} = R_1 - R_2 \times 100 / R_1$$

Where, R₁= radial growth of *Fusarium* in control

R₂= radial growth of *Fusarium* in dual inoculation

RESULTS AND DISCUSSION

Table – 1: *In vitro* antagonistic bioassay of various bacteria and fungi against *F. verticillioides*.

Sl. No.	Types of inoculum and Names of inoculum	Inhibition* in mm	
		Control (Without bioagents)	<i>F. verticillioides</i> with bio agents
Single Bacterial Inoculum (SBI), Actinomycetes			
1 A	<i>S. viridis</i>	45	34.0±0.1
1 B	<i>S. albosporus</i>	45	30.0±0.1
1 C	<i>S. cinereus</i>	45	28.0±0.1
Dual Bacterial Inoculum (DBI), Actinomycetes			
2 A	<i>S. viridis</i> and <i>S. albosporus</i>	52	15.0±0.1
2 B	<i>S. cinereus</i> and <i>S. viridis</i>	52	11.0±0.1
2 C	<i>S. albosporus</i> and <i>S. cinereus</i>	52	08.0±0.1
Single Fungal Inoculum (SFI)			
3 A	<i>T. viridae</i>	60	28.8±0.1
3 B	<i>T. harzianum</i>	60	18.8±0.2
3 C	<i>A. niger</i>	60	40.0±0.1
Dual Fungal Inoculum (DFI)			
4 A	<i>T. viridae</i> and <i>A. niger</i>	52	5.5±0.1
4 B	<i>T. harzianum</i> and <i>A. niger</i>	52	3.6±0.1
4 C	<i>T. viridae</i> and <i>T. harzianum</i>	52	2.5±0.1

*Average ± standard error from triplicate samples





Sl. No.	Types of inoculum and Names of inoculum	Control
Single Bacterial Inoculum (SBI), Actinomycetes with <i>F. verticillioides</i>		
1 A	 <p><i>S. viridis</i></p>	
1 B	 <p><i>S. albosporus</i></p>	
1 C	 <p><i>S. cinereus</i></p>	

Fig. – 1: *In vitro* antagonistic bioassay of various bacteria (SBI) against *F. verticillioides*





Sl. No.	Types of inoculum and Names of inoculum	Control
Dual Bacterial Inoculum (DBI), Actinomycetes with <i>F. verticillioides</i>		
2 A	 <p data-bbox="379 792 676 824"><i>S. viridis</i> and <i>S. albosporus</i></p>	
2 B	 <p data-bbox="411 1256 676 1288"><i>S. cinereus</i> and <i>S. viridis</i></p>	
2 C	 <p data-bbox="379 1742 699 1774"><i>S. albosporus</i> and <i>S. cinereus</i></p>	

Fig. – 2: *In vitro* antagonistic bioassay of various bacteria (DBI) against *F. verticillioides*


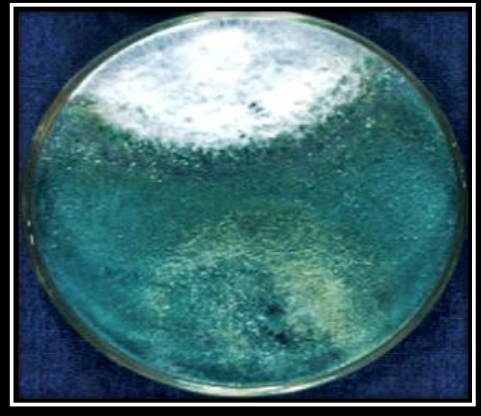


Sl. No.	Types of inoculum and Names of inoculum	Control
Single Fungal Inoculum (SFI)		
3 A	 <p data-bbox="496 779 603 808"><i>T. viridae</i></p>	
3 B	 <p data-bbox="469 1249 617 1279"><i>T. harzianum</i></p>	
3 C	 <p data-bbox="480 1715 576 1744"><i>A. niger</i></p>	

Fig. – 3: *In vitro* antagonistic bioassay of various fungi (SFI) against *F. verticillioides*




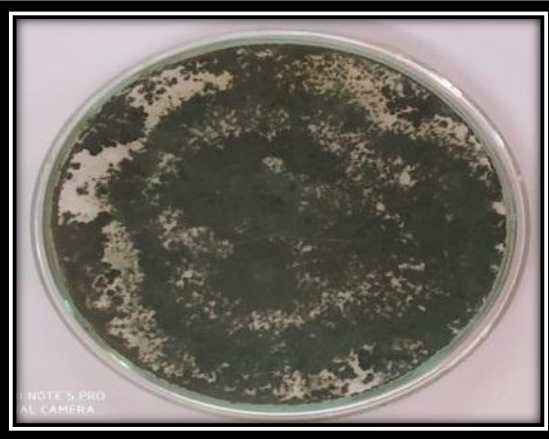
Sl. No.	Types of inoculum and Names of inoculum	Control
Dual Fungal Inoculum (DFI)		
4 A	 <p data-bbox="422 766 671 797"><i>T. viridae</i> and <i>A. niger</i></p>	
4 B	 <p data-bbox="395 1258 683 1290"><i>T. harzianum</i> and <i>A. niger</i></p>	
4 C	 <p data-bbox="375 1742 679 1774"><i>T. viridae</i> and <i>T. harzianum</i></p>	

Fig. – 4: In vitro antagonistic bioassay of various fungi (DFI) against *F. verticillioides*

Single Bacterial Inoculum (SBI) of *S. cinereus* shows more effective in controlling the growth of *Fusarium verticillioides* (28 mm in treated plate) compare to control (45 mm fungal

growth) and other two treatments i.e., *S. albosporus* and *S. viridis* (34 mm and 30 mm pathogenic fungal growth respectively). In Dual Bacterial Inoculum (DBI), the

combination of *S. albosporous* and *S. cinereus* shows more effective (08 mm pathogenic fungal growth in treated plate) compare to control (52 mm fungal growth). The other two treatments are *S. viridis* and *S. albosporus* with 15 mm pathogenic fungal growth and *S. cinereus* and *S. viridis* with 11 mm pathogenic fungal growth (Table – 1, Fig – 1 and Fig - 2).

Different isolates of *Streptomyces* spp. displayed an array of activity against pathogenic fungi, particularly *S. albosporus*. This is in conformity with the results of several studies carried out by other investigators^{3,18,19,22}. Strongly inhibited all of the pathogenic fungi with maximum percent inhibition were observed against the fungus *Penicillium digitatum* (68.6 %). Actinomycetes-fungus antagonism has been demonstrated for a variety of plant pathogens such as, *Alternaria*, *Rhizoctonia*, *Verticillium*, *Fusarium*, *Phytophthora* and *Phytium* spp.^{24,1}. The ability of isolates to inhibit the growth of fungal pathogens is implication of the volatile secondary metabolites secreted by actinomycetes. So, in the present study the potential of rhizosphere actinomycetes to inhibit the growth of pathogens has been studied. Dual-culture assays showed that some actinomycetes isolated can be developed as potential biocontrol agents. Therefore, further studies are necessary to assess the ability of the isolates to confer protection against pathogens and their role in enhancing growth and yield of plants under field conditions. Similarly, in Single Fungal Inoculum (SFI), *T. harzianum* were more effective (18.8 mm fungal growth) and in Dual Fungal Inoculum (DFI), *T. viridae* and *T. harzianum* act as more effective (2.5 mm fungal growth) against *Fusarium verticillioides* compare to controls (60 mm and 52 mm fungal growth respectively). The other treatments are *T. viridae* (28.8 mm of pathogenic fungal growth, *A. niger* (40.0 mm of pathogenic fungal growth) in SFI and *T. viridae* and *A. niger* shows 5.5 mm and *T. harzianum* and *A. niger* shows 3.6 mm of pathogenic fungal growth (Table – 1, Fig – 3 and Fig - 4).

The genus *Trichoderma* remains an economically efficient BCA that is commercially produced at a large scale and is applied against several fungal pathogens¹⁶. For filamentous fungi, the most common antimicrobial activity detection methods comprise the co-culture of two filamentous fungal strains against the pathogens. Several workers reported dual culture technique for *in vitro* evaluation of fungus to pathogenic fungi Mojica-Marin *et al.*¹², Rehman *et al.*¹⁵, Zheng *et al.*²⁵, Rahman *et al.*¹⁴. In the present investigation a pattern of 2+1 (2 – Antagonistic fungi/bacteria and 1 – Pathogenic fungi) were implemented to evaluate the effects against *Fusarium verticillioides* and combinational effects were also studied. The final interpretation of the present work reveals that DBI and DFI were more effective than SBI and SFI against *Fusarium verticillioides*.

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