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Efficacy of Fungal and Bacterial Bioagents against *Macrophomina phaseolina* under *in vitro* conditions

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ABSTRACT: Charcoal rot disease of maize caused by *Macrophomina phaseolina* (Tassi) Goid reported to be majorly responsible for increased yield losses in the regions of arid and semi arid. It is difficult to manage charcoal rot as it is soil-borne in nature. Native fungal (*Trichoderma harzianum*) and bacterial (*Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp.) bioagents were isolated from rhizosphere soil samples, collected from maize fields and they were tested for their efficacy to inhibit the mycelial growth of *Macrophomina phaseolina in vitro.* The *Trichoderma harzianum* isolate, MRTh-4 was found to be the most effective with 70.00% mycelial growth inhibition, where bacterial antagonists could able to inhibit in the range of 2.59% - 16.67% only. In conclusion, bacterial antagonists were inferior compared to *Trichoderma harzianum* in inhibiting the growth of the test pathogen and *T. harzianum* can become an important component in integrated disease management to achieve proper charcoal rot disease management in the field.

Keywords: Charcoal rot of maize, *Macrophomina phaseolina, Trichoderma harzianum,* Bacterial bioagents, Dual culture.

INTRODUCTION

After rice and wheat, maize is third most important crop of India. The total production of maize in India is 27.71 Mt from 9.02 Mha. Telangana occupies 7^{h} position in India, in terms of cultivated area of 0.54 Mha and 5th position in production with 2.08 Mt. (Indiastat, 2018-19)

One of the biggest impediments in realizing the maize crop's yield potential is diseases caused by fungi. Among them, The Post flowering stalk rot (PFSR) complex is most serious and devastating disease in maize. Internal decay and discolouration of stalk tissue reduce yield directly by limiting water and nutrient transfer and can result in lodging and plant death.

Worldwide different fungal pathogens are reported to be associated with PFSR complex but Fusarium stalk rot (*Fusarium verticillioides*), charcoal rot (*Macrophomina phaseolina*) and late wilt (*Cephalosporium maydis*) are most prevalent and destructive in nature (Khokhar *et al.*, 2014).

Among all these pathogens charcoal rot disease of maize caused by *Macrophomina phaseolina* (Tassi) Goid reported to be majorly responsible for increased yield losses in arid and semi arid regions especially where moisture stress coincides with flowering stage of the crop, particularly in rainfed maize growing areas of Telangana, Karnataka and Tamil Nadu.

It is difficult to manage charcoal rot as it is soil-borne in nature. In vitro testing gives useful preliminary information about treatment efficacy against pathogen in the shortest amount of time and so serves as a guide for subsequent field testing. It has been hypothesized that antagonistic microorganisms obtained from a crop's root or rhizosphere are better adapted to that crop and may provide better disease control than microbes isolated from other plant species (Cook, 1993).

Previous studies using different isolates of bioagents *viz., A. niger* (K) & *Trichoderma viride* II (Desai and Kulkarni, 2002); *T. viride* and *T. harzianum* (Kaur *et al*., 2012); *T. harzianum* Hyderabad isolate (Shekhar and Kumar, 2010); *A. flavus*, *A. niger* & *T. viride* (Ullah *et al*., 2011) and *T. koningi* MTCC 796 & *T. harzianum* NABII Th 1 (Gajera *et al.*, 2012) revealed mycelial inhibition of *M. phaseolina* between 51.11% - 79.29% using dual culture method. Desai and Kulkarni (2002) and Shekhar and Kumar (2010) reported sclerotial inhibition of *M. phaseolina* by different bioagents was in the range of 65% to 100%.

In field conditions, Sankar and Sharma (2001) reported that maize seed treatment with *T. viride* MR at 12 g kg^{-1} was found effective in reducing the charcoal rot incidence (12.5%) as well as increasing the yield (5007.8 kg ha^{-1}). Das *et al*. (2008) reported the use of *P. chlororaphis* SRB127 as seed treatment, reduced the charcoal rot incidence by >40%, crop-lodging by >20% and increased grain mass.

Based on the previous studies it is understood that, selection of proper isolate is very much important to achieve proper pathogen inhibition in lab as well as in field. So, in the present study, comparison of efficacy of different native bioagents in inhibiting the mycelial growth of *Macrophomina phaseolina* under *in vitro* conditions is performed.

MATERIAL AND METHODS

All the lab experiments were carried out in Department of Plant Pathology, College of Agriculture, Rajendranagar, Hyderabad, India in 2019. Efficacy of the native fungal and bacterial bioagents was evaluated against *M. phaseolina* under *in vitro* conditions by using the dual culture method (Aneja, 2018).

Collection of soil samples. Soil samples (100g each) were collected from rhizosphere of healthy maize plants of wilt effected fields for isolation of bioagents. For sampling, root system was dug out and rhizosphere soil was carefully transported in plastic bags to the laboratory. The soil samples were kept at 4°C until further use.

Isolation of Bioagents from rhizosphere soil. For isolation and maintenance of antagonist *Bacillus* spp., Nutrient agar (Madika *et al*., 2017) was used; for *Pseudomonas fluorescens*, King's B medium (KB) was used (King *et al*., 1954) and for *Streptomyces* spp., Starch casein agar (SCA) was used (Adegboye and Babalola, 2013). *Trichoderma harzianum* selective agar base (HIMEDIA) was used for the isolation of bioagent *Trichoderma harzianum* and potato dextrose agar medium was used for further maintenance.

Isolation of *Bacillus* spp. from rhizosphere soil. Soil sample (10g) was suspended in 90ml of sterile distilled water. To kill non-spore producing organisms, the soil suspension was heat shocked at 80°C for 10 minutes in a water bath (Madika *et al*., 2017). Streaking of a loopful of each soil suspension over nutrient agar medium was done.

The inoculated plates were incubated aerobically at 30°C for 24 hours and examined for colonies. *Bacillus* spp. colonies with cultural traits such as round or irregular colonies, thick and opaque colonies and cream-colored colonies were sub-cultured onto nutrient agar slants.

Isolation of *Pseudomonas fluorescens* and *Streptomyces* spp. from rhizosphere soil. In a test tube, one gram of soil sample was put into 9 ml of sterilized distilled water and properly shaken (1:10). 1 ml of suspension was transferred from the first test tube to the second test tube, which contained 9 ml sterilized distilled water (1:100), and then from the second test tube to the third test tube, which contained 9 ml sterilized distilled water (1:1000). The dilution process was repeated until 10^{-6} dilutions were achieved. For isolation of *Pseudomonas fluorescens* and *Streptomyces* spp., 10^{-6} dilutions were preferred. The diluted samples were transferred into the sterilized Petri plates at a rate of one milliliter per plate. Respective melted media i.e. KB and SCA (45°C) were poured at a rate of 20 ml per plate and spread with an inclined rotating motion of the plate. After medium solidification these plates were incubated at 27±2°C inverted for 1-2 days and well separated individual colonies were marked and picked up with sterilized loop and transferred to their respective medium.

The isolated bacterial bioagents *viz.*, *Bacillus* spp., *Pseudomonas fluorescens* and *Streptomyces* spp. were identified based on morphological, colony and microscopic characters.

Isolation of *Trichoderma harzianum* from rhizosphere soil. *Trichoderma harzianum* was isolated by serial dilution and spread plate technique (Aneja, 2018) by using *Trichoderma harzianum* selective medium (ThSM).

0.5 ml of 10^{-3} dilution was poured onto *Trichoderma harzianum* selective medium under aseptic conditions for selective isolation of *T. harzianum.* Plates were incubated for 72-96 h at 28 ± 2°C. *T. harzianum* colonies were selected and purified by hyphal tip method and maintained in PDA medium. *T. harzianum* was identified, picked on the basis of their morphological and microscopic characteristics.

Screening of antagonistic potential of *Trichoderma harzianum* isolates. The antifungal activity of eight *Trichoderma harzianum* isolates was tested against *Macrophomina phaseolina*, using dual culture technique on PDA medium (Aneja, 2018).

T. harzianum isolates and the pathogen *Macrophomina phaseolina* were grown separately on PDA for 5 days. With the use of a cork borer, five millimeter (5mm) discs of actively growing five day old cultures of test pathogen and biocontrol agents were taken. Under aseptic conditions, two discs, one each of pathogen and biocontrol agent, were placed equidistantly (60 mm) apart in each of the 90 mm Petri plates containing PDA. As a control, the plates containing PDA medium inoculated with pathogen alone were used. The plates were incubated at 28±2°C. After the control plates were completely covered by pathogen, radial growth of the bioagent and the pathogen from the centre of disc towards the centre of the plate was recorded. Each

treatment was replicated three times including control under completely randomized design (CRD).

Percent mycelial inhibition was determined by following formula

$I = (C-T/C) \times 100$

I = Per cent inhibition of mycelium

C = Radial growth of the pathogen in control (mm)

T = Radial growth of the pathogen in treatment (mm)

Screening of antagonistic potential of bacterial bioagents. Similarly, antifungal activity of twenty four bacterial bioagents (*Bacillus* spp., *Pseudomonas fluorescens* and *Streptomyces* spp.) was evaluated against *Macrophomina phaseolina*, on PDA medium using dual culture technique (Shalini *et al.,* 2017; Adhilakshmi *et al*., 2014).

From 1 cm of the plate's edge, bacterial bioagents were streaked on one side of a Petri dish with PDA media. The mycelial disc (5mm diameter) was removed from the edge of five-day-old *M. phaseolina* culture and placed on the other side of the Petri plate. Three replications were kept in a completely randomized design (CRD), with plates devoid of bioagent serving as a control. At 28 ± 2°C, the plates were incubated until the control plate was completely full and percent mycelial inhibition was calculated.

Statistical analysis. All of the treatments were repeated three times with appropriate controls, and the data was statistically analyzed using a completely randomized design (CRD) as per the standard procedures (Gomez and Gomez, 1984). The critical differences were calculated at P = 0.05. The original data was transformed to arcsine wherever necessary before analysis to bring the data into a normal distribution and the real percentage values, as well as their corresponding modified values, are shown in tables.

RESULTS AND DISCUSSION

A total of eight fungal (*Trichoderma harzianum*), eight *Bacillus* spp., eight *Pseudomonas fluorescens* and eight *Streptomyces* spp. were isolated from rhizosphere soil of healthy maize plants of wilt affected fields. The antagonistic effect of the isolated fungal and bacterial bioagents was assessed based on their ability to inhibit the pathogen growth and development under *in vitro* conditions.

All the *T. harzianum* isolates were found to produce significant reduction in pathogen growth when compared to the control (Table 1 & Plate 1). Overall, the *T. harzianum* isolate, MRTh-4 was found to be the most effective with 70.00% mycelial growth inhibition followed by MRTh-3 (65.93%) which was on par with MRTh-7 (64.81%) followed by MRTh-8 (61.11%) and rest of other treatments. However, the least mycelial inhibition was observed with MRTh-5 (42.59%).

Table 1. Antagonistic activity of *Trichoderma harzianum* against *M. phaseolina* under *in vitro* conditions.

Sr. No.	*Trichoderma harzianum* isolates	Pathogen radial growth (mm)*	Per cent inhibition over control*
1.	**MRTh-1**	47.0	47.78 (43.71) **
2.	**MRTh-2**	47.7	47.04 (43.28)
3.	**MRTh-3**	30.7	65.93 (54.26)
4.	**MRTh-4**	27.0	70.00 (56.77)
5.	**MRTh-5**	51.7	42.59 (40.71)
6.	**MRTh-6**	49.3	45.19 (42.22)
7.	**MRTh-7**	31.7	64.81 (53.61)
8.	**MRTh-8**	35.0	61.11 (51.40)
9.	**Control**	90.0	0.00 (0.00)
	SE(m)±		0.81
	CD		2.43

MRTh – Maize Rhizosphere *Trichoderma harzianum*

***** Mean of three replications

** Values in parenthesis represent angular transformed values

Plate 1. Efficacy of bioagents (*Trichoderma harzianum*) against *M. phaseolina under in vitro* conditions.

The *T. harzianum* isolates were shown to have considerable differences in their capacity to inhibit *M. phaseolina* mycelial development. Several researchers (Naik *et al*., 2000; Upmanyu *et al*., 2002; Singh *et al*., 2008) have noted this difference in isolates' ability to suppress pathogen growth, emphasizing the importance of selecting the best isolates for use as bio-control agents (Suriachandraselvan *et al*., 2004) against certain pathogens and under specific agro-climatic conditions. Furthermore, variations in the efficiency of *T. harzianum* isolates may be attributed to their genetic makeup, as these isolates may come from different ecological zones (Shalini *et al*., 2017). Antibiosis, mycoparasitism, competition for space and nutrients, and overgrowth have all been implicated for differences in inhibitory ability between isolates. (Ghaffar *et al*., 1964; Naik *et al*., 2000; Manjunatha and Naik, 2011).

All the bacterial bioagents were found to cause reduction in pathogen growth when compared to the control (Table 2 & Plate 2-4). The bioagents were found to show differences in their ability to reduce mycelial growth of *M. phaseolina*. It is evident from the Table 2, that bacterial bioagents showed mycelial inhibition in the range of 2.59% -16.67%. The bioagent MRS-5 showed highest mycelial inhibition (16.67%) which was on par with MRS-1 (13.70%) and the least inhibition shown by MRB-8 (2.59%). However, as compared to fungal antagonists, bacterial antagonists were less effective at inhibiting the growth of the test pathogen.

Plate 2. Efficacy of bioagents (*Bacillus* spp.) against *M. phaseolina under in vitro conditions.*

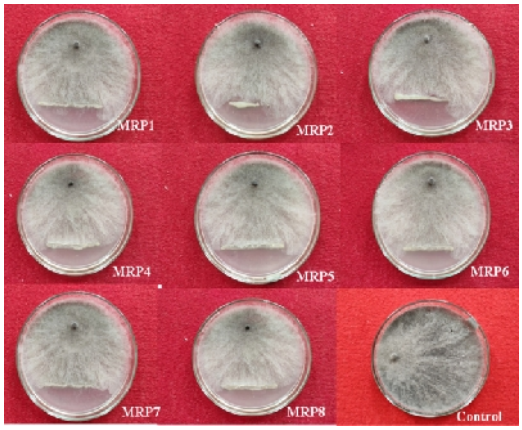

Plate 3. Efficacy of bioagents (*Pseudomonas fluorescens*) against *M. phaseolina under in vitro conditions.*

Plate 4. Efficacy of bioagents (*Streptomyces* spp.) against *M. phaseolina under in vitro* conditions.

Similar results were observed by Omoifo and Ikotun (1987) while working with maize *Macrophomina phaseolina*, that zone of inhibition was not obtained when *Bacillus* spp. was plated against *M. phaseolina* in dual culture studies.

Similarly Malleswari (2014) reported that, 43 isolates among 219 tested bacterial isolates, shown inhibition against *Macrophomina phaseolina.* Forty one bacterial antagonists inhibited in the range of 15.55% - 28.88% in dual culture. Only two isolates could able to inhibit the mycelial growth by 50.22% - 52.22%.

Kumari *et al.* (2012) reported 15.80% mycelial inhibition of *Macrophomina phaseolina* by *Pseudomonas fluorescens* in dual culture. Similarly Ali *et al*. (2014) reported that, *Pseudomonas fluorescens* inhibited *Rhizoctonia solani* mycelium in the range of 9.36% - 15.59% and fungal antagonists inhibited in the range of 41.11% - 91.85% by dual culture technique. Maruti *et al*., (2017) reported that, bacterial bioagents were able to inhibit *Rhizoctonia bataticola* by 27.87% - 34.74% compared to *Trichoderma* spp. 73.91% - 77.20% by dual culture technique.

Table 2: Antagonistic activity of bacterial biocontrol agents against *M. phaseolina* under *in vitro* conditions.

Sr. No.	Bacterial antagonists	Pathogen radial growth (mm)*	Per cent inhibition over control*
1.	**MRB-1**	85.0	5.56 (13.58)**
2.	**MRB-2**	82.7	8.15 (16.57)
3.	**MRB-3**	83.3	7.41 (15.72)
4.	**MRB-4**	85.3	5.19 (12.78)
5.	**MRB-5**	83.0	7.78 (16.16)
6.	**MRB-6**	80.3	10.74 (19.11)
7.	**MRB-7**	87.0	3.33 (8.59)
8.	**MRB-8**	87.7	2.59 (9.21)
9.	**MRPf-1**	80.3	10.74 (19.11)
10.	**MRPf-2**	80.0	11.11 (19.44)
11.	**MRPf-3**	81.7	9.26 (17.66)
12.	**MRPf-4**	82.7	8.15 (16.57)
13.	**MRPf-5**	80.3	10.74 (19.11)
14.	**MRPf-6**	81.0	10.00 (18.37)
15.	**MRPf-7**	79.3	11.85 (20.11)
16.	**MRPf-8**	80.7	10.37 (18.70)
17.	**MRS-1**	77.7	13.70 (21.67)
18.	**MRS-2**	86.3	4.07 (11.45)
19.	**MRS-3**	86.0	4.44 (11.89)
20.	**MRS-4**	86.0	4.44 (12.10)
21.	**MRS-5**	75.0	16.67 (24.08)
22.	**MRS-6**	87.0	3.33 (10.41)
23.	**MRS-7**	85.7	4.81 (12.38)
24.	**MRS-8**	79.3	11.85 (20.11)
25.	**Control**	90.0	0.00 (0.00)
	SE(m)±		1.34
	CD		3.82

MRB - Maize Rhizosphere *Bacillus* spp.; MRPf - Maize Rhizosphere *Pseudomonas fluorescens*. MRS - Maize Rhizosphere *Streptomyces* spp.

***** Mean of three replications

** Figures in parenthesis represent angular transformed values

CONCLUSION

Trichoderma harzianum isolate, MRTh-4 was found to be the most effective with 70.00% mycelial growth inhibition. Bacterial antagonists were inferior compared to *T. harzianum* in inhibiting the growth of the test pathogen. It concludes that, *T. harzianum* can become an important component in integrated disease management to achieve proper charcoal rot disease management in the field.

FUTURE SCOPE

Biological control is an important component of integrated disease management. Isolation and selection of proper bioagent with high efficacy plays a major role in success if IDM strategy for effective management of the disease in field.

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Conflict of Interest. None.

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