Original Research Article

Effectiveness of indigenous isolates of biocontrol fungi and bacteria against *Macrophomina phaseolina* causing root-rot fungus in green gram

Abstract

Green grams are the most valuable pulse crop in terms of plant-based protein, dietary fiber, and various phytochemicals. Although green gram is found susceptible to the soil-borne fungus *Macrophomina phaseolina* (Tassi) Goid, it leads to severe root-rot disease and causes a significant reduction in crop yield. Thus, the study aims to determine the effectiveness of indigenous isolates of biocontrol fungi and bacteria *viz., T. asperelloides* AMUTASPD-51, *T. asperellum* AMUTASPM-51, *T. asperellum* AMUTASPM-52, *T. asperellum* AMUTASPM-53, *T. atroviride* AMUTATROV-31, *T. harzianum* AMUTHZ-71, *T. harzianum* AMUTHZ-72, *T. harzianum* AMUTHZ-73, *T. harzianum* AMUTHZ-74, *T. hamatum* AMUTHM-31, *T. viride* AMUTVR-61, *T. viride* AMUTVR-62, *T. virens* AMUTVNS-41, *T. longibrachiatum* AMUTLONB-41, *Pochonia chlamydosporia* AMUPC-31, *Purpureocillium lilacinum* AMUPL-31, *Aspergillus niger* AMUAN-41, *Bacillus subtilis* AMUBS-80 and *Pseudomonas fluorescens* AMUPF-80 against *Macrophomina phaseolina* by using dual inoculation technique for seven days incubation at a temperature under *in-vitro* condition. All species of *Trichoderma* fungus showed high biocontrol potential to suppress the radial growth of *M. phaseolina* over control. Among the biocontrol fungi and bacteria, *T. viride* AMUTVR-61 resulted in the highest radial inhibition of *M. phaseolina* by 95.0% over control. The *T. harzianum* AMUTHZ-72 was second most effective in decreasing the radial growth by 94.2% of the pathogens, followed by *T. harzianum* AMUTHZ-71 (92.8%), *T. asperellum* AMUTASPM-53 (86.1%), and *T. harzianum* AMUTHZ-74 (83.7%) over control. However, *B. subtilis* AMUBS-80 was found to be the least effective, suppressing radial inhibition of *M. phaseolina* by 21.7% over control. The present study indicates that *T. viride* AMUTVR-61 and *T. harzianum* AMUTHZ-72 were the most significant indigenous biocontrol fungi against *M. phaseolina*. Furthermore, its application led to a substantial decrease in the soil-borne pathogen population that affects plant health, especially green gram, and adverse environmental and human effects.

Keywords: Biocontrol agents, *M. phaseolina*, *Trichoderma* spp., *Aspergillus* spp., green gram

Introduction

Pulses are an essential source of plant-based protein and staple food for the Indian people. India is one of the largest producers and consumers of pulses in the world (McDermott and Wyatt, 2017). Besides providing a healthy diet to humans, it contributes to improved soil fertility and agro-biodiversity (FAO, 2016). Among the pulses, green gram or Mung bean, *Vigna radiata* (L.) is the third most crucial pulse crop in India next to chickpea and pigeon pea (Kumar et al., 2021; Pratap et al., 2021). It is a key component in the symbiotic relationship between nitrogen-fixing rhizobium and leguminous plants. This relationship helps conserve the nitrogen components in soil and improve soil fertility for non-leguminous crops (Dudeja and Duhan, 2005; Khan et al., 2019). Green gram is a nutritionally rich, high-quality protein, carbohydrates, amino acids, vitamins, micronutrients, and low-fat content food crop (Pandey et al., 2018; Nasir et al., 2022). It is widely grown under semi-arid and sub-tropical climates and is cultivated in almost all parts of India (Mallaiah and Krishna, 2016). The crop is grown mainly in the Kharif season (Kumar et al., 2017).

Various biotic and abiotic factors have been reported to affect the growth and production of green grams so far (Nair et al., 2019; Mukhtar et al., 2021). The biotic factors include powdery mildew, mung bean yellow mosaic virus, cercospora leaf spot, anthracnose, root-rot, leaf crinkle virus, web blight, rust, and bacterial leaf blight are the most distressing agents that cause more significant reduction in crop yield (Singh et al., 2020). The root-rot fungus, *M. phaseolina*, is a highly potent and destructive pathogen that causes significant damage to the host plant at all stages of growth, including during flowering and pod formation in green gram (Dhingra and Sinclair, 1974; Shahid and Khan, 2016; Khan et al., 2019). It is a necrotrophic seed and soil-borne fungus that causes root rot disease in green gram (Dhingra and Sinclair, 1978; Rajput et al., 2023). The pathogen propagules invade urdbean and mungbean seeds and affect the germination and viability rate of the seed (Kar and Sahu, 2009; Basandrai et al., 2021). The pathogen deteriorates the stored seed quality ranging from 2% to 36% in various South Asian countries such as Bangladesh (Ali et al., 2010), Pakistan (Haider and Ahmed, 2014), India (Ashwini and Giri, 2014), and Thailand (Rahman et al., 1999). Thus, the soil-borne pathogens cause great reductions in the yield of green gram crops, ranging from 20% to 60% across various regions in India (Pandey et al., 2018).

Microbial antagonistic microorganisms have the potential to offer a cost-effective and environmentally friendly approach to controlling soil-borne phytopathogens (Harman, 2006; Thombre and Kohire, 2018; Khan et al., 2019; Iqbal and Mukhtar, 2020; Khan et al., 2023). Several biocontrol fungi and bacteria, such as *Trichoderma* species (Shahid and Khan, 2016; Khan et al., 2019), *P. chlamydosporia* (Akhtar and Siddiqui, 2009), *P. lilacinum* (Habiba et al., 2016), *A. niger* (Khan and Anwar, 2007), *B. subtilis* (Deshmukh et al., 2016) and *P. fluorescens* (Kumari et al., 2018) have been evaluated for controlling root-rot pathogens. *Trichoderma* species have evident greater effectiveness against *M. phaseolina* in field as well as laboratory conditions (Shahid and Khan, 2016; Khan et al., 2019; Iqbal and

Mukhtar, 2020). *A. niger* has been proven as a highly effective microbial antagonist against the root-rot fungus *M. phaseolina* (Shahid and Khan, 2016; Khan and Javaid, 2021). *T. atroviride*, *T. asperellum*, *T. harzianum*, *T. hamatum*, *T. koningii*, *T. polysporum* and *T. viride* are found to be effective in suppressing the mycelial growth of *M. phaseolina* in mung beans (Shahid and Khan, 2016; Khan et al., 2019; Choudhary et al., 2021). *Trichoderma* spp. exhibits mycoparasites (hindering fungal colonization), secreting lytic or cell wall-degrading enzymes (like cellulases, glucanases, proteases, chitinases, chitinases, as well as toxins, hormones, and antibiotic compounds), and nutrient competent (Vinale et al., 2014; Caulier et al., 2018). Therefore, the present study aimed to evaluate the efficacy of biocontrol fungi and bacteria *viz*., *T. asperelloides* AMUTASPD-51, *T. asperellum* AMUTASPM-51, *T. asperellum* AMUTASPM-52, *T. asperellum* AMUTASPM-53, *T. atroviride* AMUTATROV-31, *T. harzianum* AMUTHZ-71, *T. harzianum* AMUTHZ-72, *T. harzianum* AMUTHZ-73, *T. harzianum* AMUTHZ-74, *T. hamatum* AMUTHM-31, *T. viride* AMUTVR-61, *T. viride* AMUTVR-62, *T. virens* AMUTVNS-41, *T. longibrachiatum* AMUTLONB-41, *P. chlamydosporia* AMUPC-31, *P. lilacinum* AMUPL-31, *A. niger* AMUAN-41, *B. subtilis* AMUBS-80 and *P. fluorescens* AMUPF-80 to manage root-rot fungus caused by *M. phaseolina* in green gram. This study also showed the isolation of native biocontrol fungi and bacteria from soil-borne pathogens to substitute chemical fungicides for soil-borne pathogens. This study enlightens the incorporation of plant and microbial-based materials in the disease management module rather than using synthetic agrochemicals in soil fertilization and crop protection.

Materials and methods

Isolation and identification of root-rot fungus

The root-rot fungus, *Macrophomina phaseolina* was isolated from the infected roots of the green gram. The infected root sample was cut into small pieces (2-5mm) and surface sterilized by dipping in 1% sodium hypochlorite (w/v) for 30 seconds and then rinsed twice with distilled water. The pieces were dried on sterilized absorbent tissue paper and placed onto a petri dish containing solidified potato dextrose agar (PDA). The inoculated plates were kept at 28±2°C in an incubator for a week. After incubation, the fungus colonies were examined under a microscope and compared to *Macrophomina phaseolina* characteristics. Hence, the root-rot fungus was examined based on its morphological and cultural characteristics.

Isolation and identification of biocontrol fungi and bacteria

The biocontrol fungi, *viz*., *Trichoderma* species, *P. chlamydosporia*, *P. lilacinum* and *Aspergillus niger* were isolated from several green gram field soil using serial dilution method on Trichoderma selective medium, Corn Meal Agar, and Aspergillus selective medium, respectively. The soil sample of 10 gm was collected separately from each sample and mixed with 90 ml of double distilled water (DDW) in a 100 ml Erlyn Mayer flask. The flask containing soil solution was homogenized using a shaker for 10 minutes. After that, the flask was stand in a laminar flow for 10 minutes to settle down heavy particles. For soil

dilution, 1 ml of the soil solution was pipetted into a culture tube containing 9 ml DDW, shaken, and marked as 10^{-2} dilution. This process was repeated until 10^{-4} or 10^{-6} dilution level was achieved. For isolation of *Trichoderma* species, *P. chlamydosporia*, *P. lilacinum* and *Aspergillus* species, 100µl of dilution 10^{-4} was spread on solidified Trichoderma selective media (TSM), Corn Meal Agar (CMA) and Aspergillus selective media (ASM), respectively. The plates were sealed with parafilm tape and incubated at 28±2℃ for ten days. *Trichoderma* colonies from TSM, *P. chlamydosporia,* and *P. lilacinum* colonies from CMA and *A. niger* colonies from ASM were sub-cultured on solidified PDA under sterilized conditions. The plates were incubated at 28±2℃ for ten days. After incubation, the isolates of *Trichoderma* species, *P. chlamydosporia, P. lilacinum* and *A. niger* were processed for morphological identification based on colony size, mycelium, conidiation colour, pattern and colour of the medium. The microscopic characteristics such as conidiophores, conidia, phialides, or mycelial structures were examined under 40× magnifications.

The biocontrol bacteria *viz., B. subtilis* and *P. fluorescens* 100µl 10^{-6} dilution from were spread onto solidified Nutrient Agar (NA) medium in Petri plates under a flame in a Laminar flow. The inoculated Petri plates were sealed with parafilm tape and incubated for 24 hours at 37.8°C in a BOD incubator. After incubation, streaking with a single colony was done on NA medium in Petri plates. The colonies were examined for colour, size, shape, gram response, and cell shape to confirm *B. subtilis* and *P. fluorescens* (Brown, 1939).

***In-vitro* efficacy of biocontrol fungi and bacteria against root-rot fungus**

The efficacy of nineteen indigenous isolates of biocontrol fungi and bacteria *viz., T. asperelloides* AMUTASPD-51, *T. asperellum* AMUTASPM-51, *T. asperellum* AMUTASPM-52, *T. asperellum* AMUTASPM-53, *T. atroviride* AMUTATROV-31, *T. harzianum* AMUTHZ-71, *T. harzianum* AMUTHZ-72, *T. harzianum* AMUTHZ-73, *T. harzianum* AMUTHZ-74, *T. hamatum* AMUTHM-31, *T. viride* AMUTVR-61, *T. viride* AMUTVR-62, *T. virens* AMUTVNS-41, *T. longibrachiatum* AMUTLONB-41, *P. chlamydosporia* AMUPC-31, *P. lilacinum* AMUPL-31, *A. niger* AMUAN-41, *B. subtilis* AMUBS-80 and *P. fluorescens* AMUPF-80 against *M. phaseolina* was evaluated under *in-vitro* condition by following the dual culture plate method (Dennis and Webster, 971). The biocontrol fungi, bacteria and test pathogen (*M. phaseolina*) of 5 mm diameter disc were taken from seven days old cultures and placed oppositely towards the periphery of the petri plates containing PDA media. The antagonistic activity of biocontrol fungi and bacteria was observed against the test fungus by measuring the percent inhibition of mycelial growth of the pathogenic using equation no. 1. The dual culture plates were maintained in five replications and incubated at 28±2°C in a BOD for five days.

$$PI = \{(C - T) / C\} \times 100 \ldots\ldots \text{Eq. (1)}$$

Where,

I = Per cent inhibition

C = Control (radial growth)

T = Treatment (radial growth)

Statistical analysis

The table data were presented in mean values of five replications of each treatment using MS Excel 2021. The data on the colony diameter (mm) of pathogen and biocontrol fungi and bacteria were analyzed through single-factor ANOVA. The single-factor ANOVA to mycelial growth inhibition (%) was evaluated in terms of Fisher's least significant difference (LSD), coefficient of variation (CV), and standard error of the mean (SEM) at the probability level, $P \leq 0.05$. The data on percent growth inhibition is presented as a box plot with one-way ANOVA and represents the Tukey test using Origin-Pro software, 2024. The statistical significance between the treatments was determined by the Tukey HSD test at the probability level, $P \leq 0.05$, using R software (R Development core team 2014).

Results

Antagonistic effects of biocontrol fungi against root-rot fungus, *in-vitro*,

The result of the present study revealed that the nineteen indigenous isolates of biocontrol fungi and bacteria *viz.*, *T. asperelloides* AMUTASPD-51, *T. asperellum* AMUTASPM-51, *T. asperellum* AMUTASPM-52, *T. asperellum* AMUTASPM-53, *T. atroviride* AMUTATROV-31, *T. harzianum* AMUTHZ-71, *T. harzianum* AMUTHZ-72, *T. harzianum* AMUTHZ-73, *T. harzianum* AMUTHZ-74, *T. hamatum* AMUTHM-31, *T. viride* AMUTVR-61, *T. viride* AMUTVR-62, *T. virens* AMUTVNS-41, *T. longibrachiatum* AMUTLONB-41, *P. chlamydosporia* AMUPC-31, *P. lilacinum* AMUPL-31, *A. niger* AMUAN-41, *B. subtilis* AMUBS-80 and *P. fluorescens* AMUPF-80 showed inhibitory effects against *M. phaseolina* (Fig. 2). The indigenous isolates of biocontrol fungi and bacteria effectively suppressed the mycelial growth of test pathogens compared to the untreated control (Fig. 2). Among biocontrol fungi and bacteria, *T. viride* AMUTVR-61 showed higher mycelial inhibition of 95.0% of the test pathogen over untreated control ($P \leq 0.05$; Table 1, Fig. 1). Next in order was *T. harzianum* AMUTHZ-72, which showed mycelial inhibition of 94.2%, followed by *T. harzianum* AMUTHZ-71 and *T. asperellum* AMUTASPM-53 exhibited a percent inhibited the test pathogen by 92.8% and 86.1% compared to the control ($P \leq 0.05$; Table 1, Fig. 1). Similarly, *T. harzianum* AMUTHZ-74 was also significantly suppressed the mycelial growth of *M. phaseolina* by 83.7% followed by *T. atroviride* AMUTATROV-31 (72.3%) and *T. asperelloides* AMUTASPD-51 (70.4%) over control ($P \leq 0.05$; Table 1; Fig. 1). The treatment of *Bacillus subtilis* AMUBS-80 showed relatively lower effectiveness, as indicated by an inhibition zone of 21.7% against the test pathogens ($P \leq 0.05$; Table 1; Fig. 1).

Discussion

Green gram is one of the important pulse crops, but its productivity is considerably affected by biotic and abiotic factors in India compared to other countries (Kumar et al., 2021; Pratap et al., 2021). The root-rot fungus *Macrophomina phaseolina* is one of the most economically significant pathogens of green gram that has a negative impact on plant yield and production (Ikram and Dawar, 2013; Shahid and Khan, 2016; Pandey et al., 2018; Khan et al., 2019). The pathogen, *M. phaseolina* infects leaves, pods, and roots, resulting in defoliation or blighted appearance of leaves (Singh et al., 2020; Basandrai et al., 2021). The present study found that all indigenous isolates of biocontrol fungi and bacteria significantly suppressed the mycelial growth of *M. phaseolina in vitro*. The dual inoculation test revealed biocontrol fungi and bacteria, *T. viride* AMUTVR-61 and *T. harzianum* AMUTHZ-72 showed maximum suppression of the colonization *M. phaseolina* pathogen followed by *T. harzianum* AMUTHZ-71 and *T. asperellum* AMUTASPM-53. Similarly, Khan et al. (2019) determined that *T. harzianum* and *T. viride* were significantly inhibited by 70-73% colonization of *M. phaseolina*. *T. hamatum* significantly decreased the *M. phaseolina* mycelial growth by 76.3% (Khan et al., 2019; Iqbal and Mukhtar, 2020). Similarly, several other researchers reported an inhibitory effect of *T. hamatum, T. virens, A. niger,* and *T. longibrachiatum* against *M. phaseolina* in terms of its radial growth suppression (Singh et al., 2012; Khan et al., 2019; Choudhary et al., 2021). The plates that were dual inoculated with both *Trichoderma* spp. and the pathogen showed significant competition (Harman, 2006). The inhibitory effect may suppress the pathogens through various mechanisms, including mycoparasitism (Weindling 1932), antibiotics (Vinale et al., 2014; Ajitha and Lakshmidevi, 2010), and competition for nutrients, space (Hjeljord et al., 2000) and induce systemic resistance (Vinale et al., 2008). Another treatment of *T. virens* AMUTVNS-41, *T. longibrachiatum* AMUTLONB-41, *P. chlamydosporia* AMUPC-31, *P. lilacinum* AMUPL-31, *A. niger* AMUAN-41, *B. subtilis* AMUBS-80 and *P. fluorescens* AMUPF-80 significantly decreased the radial growth of *M. phaseolina.* Likewise, *T. harzianum, T. virens, T. fasciculatum, T. asperellum, T. viride, P. chlamydosporia*, *P. lilacinum, B. subtilis,* and *P. fluorescens* exhibited a significant antagonistic impact against the *M. phaseolina* (Akhtar and Siddiqui, 2009; Habiba et al., 2016; Deshmukh et al., 2016; Kumari et al., 2018; Khan et al., 2019; Choudhary et al., 2021). The results of the experiments revealed that the biocontrol fungi and bacteria, *Trichoderma* spp., *P. lilacinum, P. chlamydosporia, A. niger, B. subtilis* and *P. fluorescens* exhibited mycoparasitism and antibiosis as potential mechanisms for parasitizing and suppressing pathogens. These mechanisms have the potential to control dry root rot disease effectively.

Conclusion

The present study concludes that indigenous isolates of biocontrol fungi and bacteria significantly suppress the mycelial growth of *Macrophomina phaseolina in vitro*. The dual inoculation test revealed that biocontrol fungi, *T. viride* AMUTVR-61, *T. harzianum* AMUTHZ-72, T. harzianum AMUTHZ-71, *T.*

asperellum AMUTASPM-53, and *T. harzianum* AMUTHZ-74 showed maximum percent of colony growth inhibition. Biocontrol fungi and bacteria are effective alternatives to synthetic fertilizers and fungicides. The soil microbiome rich in *Trichoderma* and *Aspergillus* species is the best biological agent in maintaining soil fertility, promoting plant growth, and reducing soilborne pathogen colonization. The multifaceted effects of biocontrol fungi attract researchers' attention to the improvement of soil nutrient management practices and crop production. Besides the biocontrol potential of beneficial microbes, they help attain economic sustainability, increase renewability, conserve biodiversity, and promote environmental safety at both farmers' and commercial levels.

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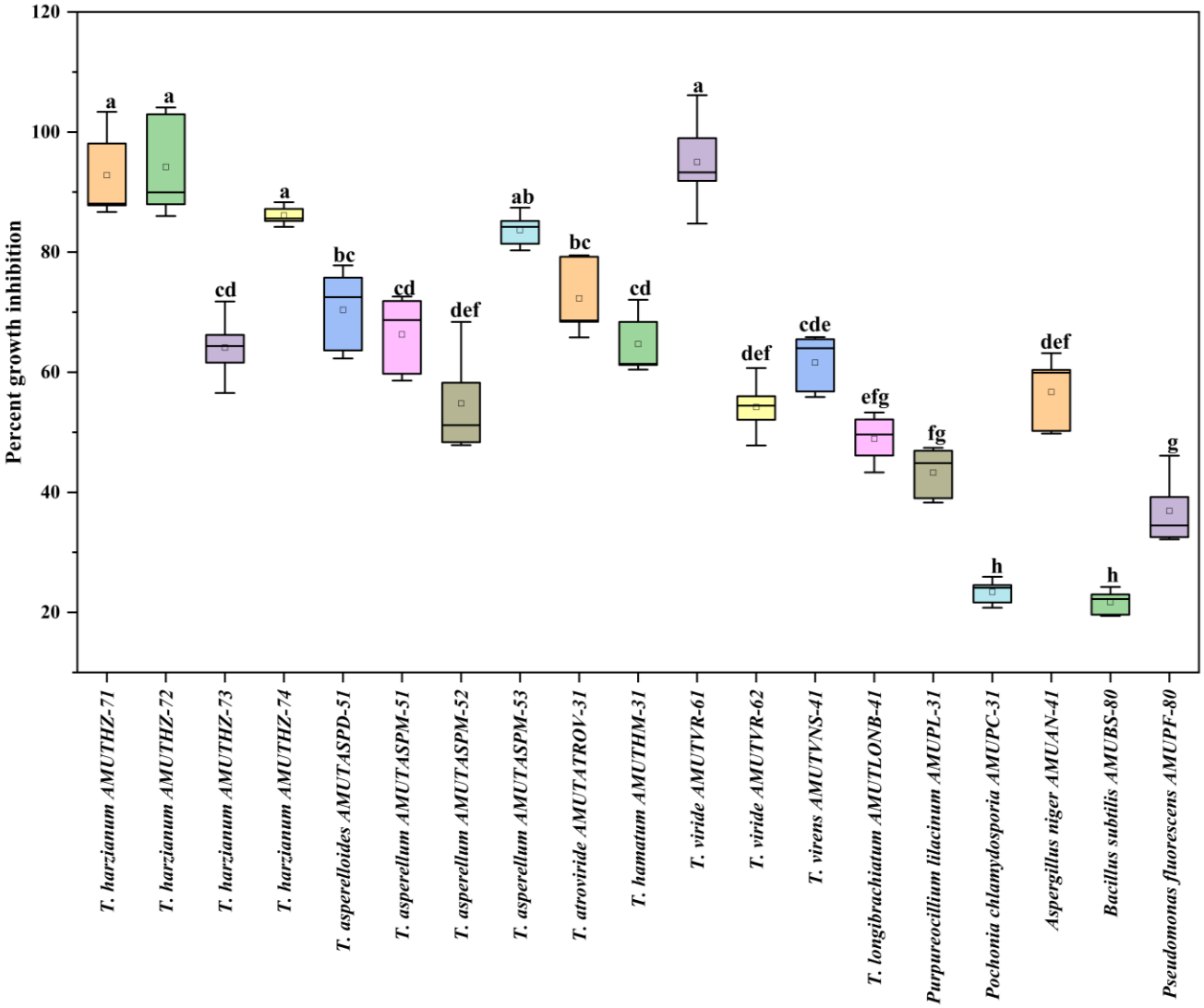

Figure 1. *In-vitro*, the effect of biocontrol fungi and bacteria on the percent growth inhibition of *Macrophomina phaseolina* AMUMP-2. Different alphabets are indicated significantly different at $P \leq 0.05$ according to Tukey test. Error bars show standard deviation

Table 1. *In-vitro*, the effect of biocontrol fungi and bacteria on the colonization of *Macrophomina phaseolina* AMUMP-2.

Biocontrol agents		Colony Diameter (mm)		Growth inhibition (%)
		***Macrophomina phaseolina* AMUMP-2**	**Biocontrol agents**	
Control		90.0^{a}	-	-
T. asperelloides AMUTASPD-51		26.6^{hi}	63.4^{cde}	70.4^{bc}
T. asperellum AMUTASPM-51		30.3^{ghi}	75.2^{bc}	66.3^{cd}
T. asperellum AMUTASPM-52		40.7^{ef}	49.3^{fg}	54.8^{def}
T. asperellum AMUTASPM-53		12.8^{kl}	92.2^{a}	86.1^{a}
T. atroviride AMUTATROV-31		24.9^{ij}	65.1^{cd}	72.3^{bc}
T. harzianum AMUTHZ-71		6.5^{kl}	83.5^{ab}	92.8^{a}
T. harzianum AMUTHZ-72		5.2^{l}	84.8^{ab}	94.2^{a}
T. harzianum AMUTHZ-73		32.3^{fghi}	57.7^{def}	64.1^{cd}
T. harzianum AMUTHZ-74		15.4^{jk}	94.5^{a}	83.7^{ab}
T. hamatum AMUTHM-31		31.8^{fghi}	58.2^{def}	64.7^{cd}
T. viride AMUTVR-61		4.5^{l}	85.5^{ab}	95.0^{a}
T. viride AMUTVR-62		41.2^{ef}	48.8^{fg}	54.2^{def}
T. virens AMUTVNS-41		34.6^{fgh}	55.4^{def}	61.6^{cde}
T. longibrachiatum AMUTLONB-41		46.0^{de}	59.4^{def}	48.9^{efg}
Pochonia chlamydosporia AMUPC-31		68.9^{b}	21.1^{ij}	23.4^{h}
Purpureocillium lilacinum AMUPL-31		51.0^{cd}	39.0^{gh}	43.3^{fg}
Aspergillus niger AMUAN-41		39.0^{efg}	51.0^{efg}	56.7^{def}
Bacillus subtilis AMUBS-80		70.5^{b}	19.5^{j}	21.7^{h}
Pseudomonas fluorescens AMUPF-80		56.8^{c}	33.2^{hi}	36.9^{g}
LSD $P \leq 0.05$		5.23	6.86	7.33
CV		11.41	9.11	9.28
SEM		17.32	29.73	33.87
ANOVA				
Treatment	Df	19	18	18
	Sum Sq	50255	44898	43682
	Mean Sq	2645	2494	2426.8
	F value	152.7**	83.88**	71.63**
Residuals	Df	80	76	46
	Sum Sq	1386	2260	2575
	Mean Sq	17.3	29.7	33.9

Each values are means of five replicates. Values followed by different alphabets within column are significantly different at $P \leq 0.05$ according to Tukey test. **F values are significant at $P \leq 0.05$.