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ANTAGONISM OF SELECTED FUNGAL SPECIES AGAINST *MACROPHOMINA PHASEOLINA* (TASSI) GOID, CAUSING CHARCOAL ROT OF MUNGBEAN

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Abstract

Macrophomina phaseolina (Tassi) Goid is a destructive soil-borne fungus that infect several crops and cause considerable yield losses. In this work, *M. phaseolina* was isolated from symptomatic munbean plants and was identified on the basis of morphological features. Morphological identification was further confirmed by the amplification of ITS regions which exhibited 99 to 100% identity with sequences of *M. phaseolina* present in previously published literature. In antagonistic assays, strains of *Trichoderma harzianum*, *Penicillium* spp. and *A. flavus* significantly inhibited the mycelial growth in *M. phaseolina* except *A. niger*. Fungal agents with promising antagonism in dual culture were further investigated for disease suppression and plant growth promotion effects on five selected mungbean varieties in net house. Of all the tested fungal agents, *T. Harzianum* and *Penicillium* spp. significantly curbed the disease severity percentage, and showed positive effects on seed germination percentage and other plant growth parameters. An in-depth study of these fungal antagonists needs to be carried out which can lead to develop a better substitute to chemical formulations.

Key words: Antagonism, *Macrophomina phaseolina,* Mungbean, Biocontrol, Plant growth promotion.

Introduction

Mungbean (*Vigna radiata* L.) Wilczek, is cultivated as a pulse crop in diverse agroecological zones. The yield of this crop in Pakistan is very low as compared to other mungbean growing countries (Iqbal & Mukhtar, 2014). Various abiotic and biotic factors attribute to lowering the crop yield. Mungbean is susceptible to the attack of about twenty six diseases around the world (Charles, 1978). Among the biotic factors, *M. phaseolina* (Tassai) Goid., cause charcoal rot in various field crops resulting into grain yield losses and adversely affect the mungbean crop (Kumari *et al.*, 2012). Due to seed and soil-borne nature, *M. phaseolina* is reported to infect nearly 500 plant species in more than 100 plant families round the globe (Mihail & Taylor, 1995). Whereas, 67 host plant species of this devastating disease causing agent are recorded from Pakistan. *M. phaseolina* shows variability in physiology (Mihail & Taylor, 1995), morphology, pathogenicity (Rayatpanah *et al.*, 2012) and genetics (Jana *et al.*, 2005; Reyes-Franco *et al.*, 2006; Aboshosha *et al.*, 2007; Prabhu *et al.*, 2012) which make this pathogen well adapted to persist in variety of agro-ecological zones.

Greater variability among the population of *M. phaseolina* makes management strategies complicated and in some cases ineffective. Chemical control is very efficient, yet shows residual effects that result in environmental pollution, and also pose serious threat to the human health (Kumari, 2012). As, chemical control of this pathogen is challenging due to sustained persistence in soil therefore, antagonistic agents were given preference as they impede the pathogen growth by striving in competition for space, nutrients and also by secreting specific enzymes that kill the pathogens by destroying their cell components (Agrios, 2005).

Various fungal agents such as *Trichoderma* spp., *Penicillum* spp. (Alam *et al.*, 2010), *Aspergillus* spp. and certain rhizobacteria *viz*., *Pseudomonas* spp. (Rajeswari & Kannabiran, 2011) have been disclosed to show plant growth promotion and antagonism against *M. phaseolina,* and various other soil borne fungal agents *viz., Fusarium oxysporum* and *Pythium* spp. (John *et al.*, 2010; Hyder *et al*., 2021), *Phytophthora* spp. (Hyder *et al*., 2020), *Verticillium* spp. (Ruano-Rosa *et al.*, 2015), *Armillaria* spp. (Pellegrini *et al.*, 2014) and *Rhizoctonia* spp. (Daryaei *et al.*, 2016). There is a need to explore efficient biocontrol agent based pesticides as better substitute to synthetic agrochemicals. The current work is focused on exploring the antagonistic activities of some selected fungal strains against *M. phasiolina* and plant growth promotion effects on mungbean. This research can be helpful to develop a nontoxic solution as one of the component of integrated disease management (IDM) strategy by exploring the antagonism of tested fungal strains against *M. phaseolina* and other noxious pathogens. There is a need to carry out in depth research to develop antagonistic fungal agent based bio-formulations as an alternative to toxic synthetic chemicals.

Materials and Methods

Isolation, culturing and morphological identification of *M. phaseolina*: A total of fifteen *M. phaseolina* strains were isolated from the infected mungbean plant samples. Symptomatic plants presenting charcoal rot symptoms were obtained from Faisalabad (31.4504° N, 73.1350° E) Pakistan. Infected shoots bearing pycnidial growth, showing blackish brown discoloration (Sattar and Hafiz, 1952), and roots carrying dark-black microsclerotia (Dubey & Upadhyay, 2001) were collected in zipped polythene bags. *M. phaseolina* was isolated by aseptical excision of small epidermal sections from each infected shoot and root sample, and was transferred on Petri plates topped with potato dextrose agar (PDA, Sigma Aldrich, USA) medium. Petri plates were incubated at 26 ± 1°C for seven days under dark conditions. Purification of fungal

cultures was performed using hyphal tip method (Toussoun & Nelson, 1968) onto glucose and agar medium, and kept in dark at 26±1°C for a week. To separate the sclerotial bodies, a small portion of actively growing fungal mycelia was agitated on a glass slide bearing a water drop. Seclerotial bodies were transferred on solidify agar media, and incubated at 26±1°C for pure culture development. Micromorphological studies (i.e. colony growth pattern, radial colony growth, sclerotial size and weight) helped to identify the pure cultures as *M. phaseolina* as described (Nelson *et al.*, 1983) (Fig. 1). Pure culture of *M. phaseolina* was stored on PDA media in 10 mL culture tubes at 4°C (Dhingra & Sinclair, 1973).

Pathogenicity assay: The virulence of the fungal isolates was checked according to Koch's postulates. Pathogenicity of fifteen fungal isolates was tested on a susceptible mungbean cultivar NCM-257-10 (Khan & Shuaib, 2007) in growth room conditions in a repeated experiment. Mungbean seeds were treated with 1% sodium hypochlorite (NaClO) solution for 3 minutes, rinsed 3 times with sterilized distilled H_2O, and surface dried on sterilized filter paper under aseptic conditions. Sterilized seeds (10 seeds/plate) were placed on Petri plates containing one week old culture of *M. phaseolina*. Sterilized seeds placed on wet filter paper without fungal inoculum were used as control. All Petri dishes were incubated at 26 ± 1°C in dark for 12 days. Each treatment was comprised of three Petri plates with three replications. Percentage seed infection was recorded by observing the infected seeds to the total number of seeds/plate x 100, while disease severity was calculated by following the disease severity scale presented by (Manici *et al.*, 1992).

Molecular characterization of *M. phaseolina*: To confirm the morphological identification, genomic DNA was extracted from four highly virulent *M. phaseolina* isolates (MP-z11, MP-018, FSD-MB15 and FSD-MB09) using the standard protocol of PrepMan fungal DNA extraction kit manufacturers. The internal transcribed spacer (ITS) region was amplified by using ITS1 and ITS4 primers. Genomic DNA was amplified in polymerase chain reaction (PCR). A total reaction volume of 100 µl (deionized water 78 µl; Taq polymerase buffer 10 µl 10X; Taq polymerase 1 µl of 1 U; dNTPs 6 µl 2 mM; primers 1.5 µl of 100 mM) was prepared. PCR reaction conditions were; initial denaturation of DNA at 94°C for 5 min; 35 cycles of denaturation of DNA at 94°C for 30 s; annealing at 61°C for 30 s; extension of the product at 70°C for 2 minutes, and the final product extension at 72°C for 7 min. Amplified products were gel purified by using Gene Jet PCR Purification Kit (Thermo Scientific), and were sent to Macrogen Korea for sequence analysis. Obtained sequences were firstly aligned in sense and anti-sense directions using BioEdit software (Hall *et al.*, 2003), and the final sequences were compared with the relevant nucleotide sequences already available in GenBank repository by using Basic Local Alignment Search Tool (BLAST). Final sequences were submitted to National Center for Biotechnological Information (NCBI; https://www.ncbi.nlm.nih.gov/) under the accessions; MH371345, MH371315, MH371335, and MH371316. Phylogenetic tree was constructed in MEGA software using maximum likelihood method with 1000 bootstrap repeats.

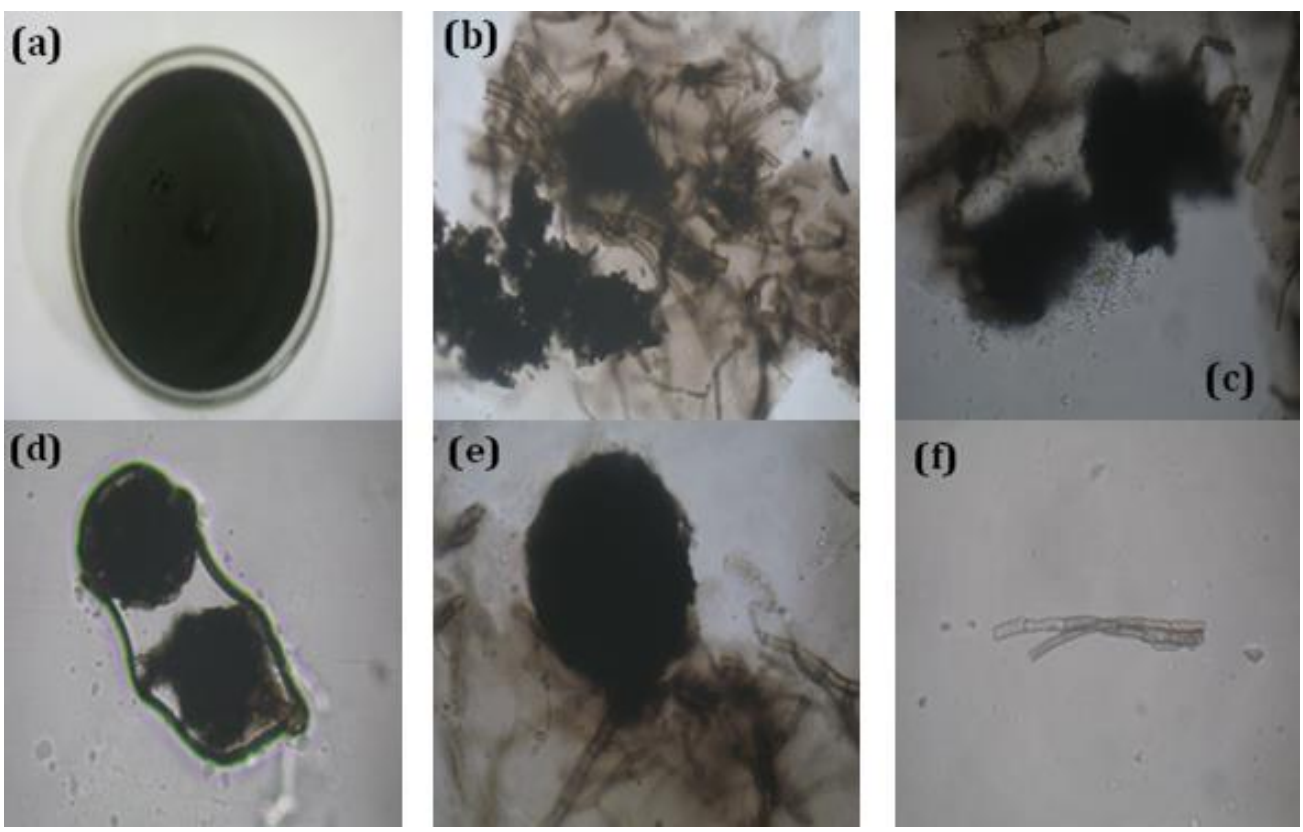

Fig. 1. Pure culture of *M. phaseolina* isolated from symptomatic mungbean plants on PDA medium. (a) Pure culture of *M. phaseolina* (Dense); (b) Mycelial mates + Sclerotial bodies; (c, d, e) Sclerotial bodies (f) Hyphal structure under microscope.

Fungal antagonists: Fungal isolates of *Trichoderma harzianum* (Trh1, Trh2, Trh3) and *T. varidae* (Trv1, Trv2, Trv3) were recovered from the soil samples taken from healthy mungbean fields by using serial dilution method (Gill *et al.*, 2009). In particular, soil suspension was diluted from 10^{-1} to 10^{-7} folds in sterilized distilled water, and 100 µl from each dilution was transferred on Petri dishes containing PDA medium (pH 6) supplemented with rose bengal (0.03g/l), chloramphenicol (0.4g/l), and streptomycin sulfate (0.03g/l), and incubated for seven days at 26 ± 2ºC. Obtained fungal isolates were identified by consulting taxonomic key for the genus *Trichoderma* (Rifai, 1969) whereas, the pure cultures of *Aspergillus niger*, *A. flavus* and *Penicillum* spp. were obtained from the Department of Plant pathology, UAF Pakistan, and stored at 26 ± 2 ºC till further use in the experiments.

Antagonistic Assay of fungal isolates against *M. phaseolina*: Antagonistic activity of selected fungal isolates was evaluated against Mp by dual culture assay (Elad *et al.*, 1986) in repeated experiments. In particular, five days old actively growing cultures of virulent strain of *M. Phaseolina* - FSD-MB09 and test fungal antagonists were counter grown aseptically onto the top of PDA media containing Petri plates. The control plates contained only *M. phaseolina* culture. The assay was performed in five repeats for each treatment. Petri dishes were gravitated at 26 ± 2°C, and zone of inhibition was measured 48, 72, 96 and 120 hours after inoculation (HAI) from each Petri plate. Percent mycelial growth inhibition was recorded by using formula presented by (Lokesha & Benagi, 2010).

$$I = \frac{Cf - Tf}{Cf} \times 100$$

I = Percent mycelial growth inhibition, Cf = fungal growth in control, Tf = fungal growth in treatment.

Evaluation of disease suppression and plant growth promotion effect of fungal isolates against *M. phaseolina*: Fungal antagonists with promising antagonistic potential in dual culture assays were further evaluated for disease suppression, and plant growth promotion traits on five mungbean cultivars in net house experiments. In brief, ten seeds of each cultivar were firstly immersed in 2.5% NaOCl solution, followed by three consecutive washings in sterilized distilled water. Mungbean seeds after surface sterilization were dipped in spore suspension (10^5 spores/ml) of test biocontrol agents for 30 minutes, and treated seeds were dried on paper towel. Tween-80 was added in each suspension before seed dipping to enhance the chances of spore adhesion to the seeds. Coated seeds were grown in 1.5 L plastic pots carrying soil pre-infested with *M. phaseolina* culture (3 g/kg soil). Pots without *M. phaseolina* culture were kept as control. All the pots were kept in net house at 26 ± 2°C in five replications for each treatment. All other cultural practices were maintained same for each treatment. Germinating seed percentage, plumule length, radical length, vigor index and biomass were recorded thirty days after sowing the seeds. Vigor index was calculated by using the formula presented by (Zhao *et al.*, 2016).

Vigor index (VI) = (Average root length + Average shoot length) x Germinating seed percentage

Statistical analysis

All the experiments were repeated twice to confirm the results, and mean values were calculated. Linear model was selected to study analysis of variance (ANOVA) for collected data using STATISTIX 8.1, Copyright (C) 1985-2005, analytical software. All pair wise comparison was carried out to study the differences among means by least significant difference test at 5% probability level.

Results

In our study, fifteen isolates of *M. phaseolina* were recovered from symptomatic shoot and root samples of mungbean plants. Pure cultures of these collected isolates were identified on the basis of morphological feature (Table 1). All the tested isolates showed variability in radial growth ($F = 9.79$; Df 14, 30; $p<0.05$) ranging from 29.94 mm to 84.18 mm. Maximum colony diameters 84.18 and 75.68 mm were recorded in MP-c12 and MP-018, while minimum colony diameters were recorded 29.94 and 33.03 mm in MP-1322 and MP-023 respectively. No significant difference was recorded among the isolates in terms of sclerotial size, and weight. Sclerotial size ranged from 14.54 to 27.71 μm while sclerotial weight was ranged from 0.137 to 0.210 mg. Out of fifteen, thirteen isolates showed denser colony growth on PDA medium seven days after incubation, while two isolate MP-a and MP-011 showed feathery growth pattern.

In pathogenicity assay, variations among the isolates virulence were observed when tested on susceptible mungbean variety NCM 257-10. The percentage disease infection due to *M. phaseolina* varied from 28.8 to 70.5 % by all the tested fungal isolates (Fig. 2). Among all the tested isolates of *M. phaseolina*, two isolates FSD-MB09 and FSD-MB15 displayed significantly high disease infection 70.5% and 67% followed by MP-z11 (56.7%), and MP-018 (55.6%) whereas, isolates MP-221, MP-412, MP-2k1 and MP-1322 were found less virulent.

Four most virulent isolates of *M. phaseolina* MP-z11, MP-018, FSD-MB15 and FSD-MB09 were also identified on the basis of molecular analysis (Fig. 3). All the four accessions showed 99 to 100% sequence homology with already available GenBank accessions of *M. phaseolina* MH168332, KC513786, HQ660594 and HQ660589 isolates reported from India and China. Phylogenetic tree of obtained sequences and few retrieved sequences from NCBI database showed that two isolates; MP-z11 and MP-018 were genetically identical with Indian isolates (accessions; MH168332, KC513786) while the other two isolates FSD-MB15 and FSD-MB09 showed maximum sequence homology with the Chinese isolates (accessions; HQ660594, HQ660589) of *M. phaseolina*.

Table 1. Morphological characters of *M. phaseolina* strains isolated from infected mungbean plants.

Isolates	Radial growth (mm)	Sclerotial size (μ m)	Sclerotial weight (mg)	Colony growth pattern
MP-018	75.68 ± 0.39 b	27.71 ± 0.74 a	0.187 ± 0.01 abc	Dense
MP-c12	84.18 ± 1.15 a	27.02 ± 0.44 a	0.210 ± 0.02 a	Dense
MP-34k	70.03 ± 3.61 bcd	25.8 ± 2.15 a	0.207 ± 0.02 ab	Dense
MP-d53	45.01 ± 1.36 f	25.71 ± 1.36 a	0.153 ± 0.00 bc	Dense
MP-412	67.38 ± 1.14 cd	25.05 ± 0.98 a	0.177 ± 0.02 abc	Dense
MP-023	33.03 ± 1.24 gh	24.12 ± 2.07 ab	0.157 ± 0.01 abc	Dense
FSD-MB15	55.85 ± 2.80 e	23.86 ± 1.02 ab	0.207 ± 0.03 ab	Dense
MP-1322	29.94 ± 3.89 h	20.87 ± 2.29 bc	0.155 ± 0.01 bc	Dense
MP-011	63.90 ± 0.88 d	20.21 ± 0.59 bcd	0.153 ± 0.01 bc	Feathery
MP-221	52.94 ± 1.18 e	19.06 ± 0.79 cde	0.157 ± 0.02 bc	Dense
MP-a	36.41 ± 1.98 g	18.6 ± 1.38 cde	0.143 ± 0.01cd	Feathery
FSD-MB09	72.98 ± 1.20 bc	18.31 ± 0.60 cdef	0.150 ± 0.02 c	Dense
MP-z11	53.78 ± 4.25 e	16.88 ± 2.43 def	0.137 ± 0.04 cd	Dense
MP-13t	54.56 ± 0.33 e	15.99 ± 0.37 ef	0.153 ± 0.02 bc	Dense
MP-2K1	53.13 ± 1.63 e	14.54 ± 0.39 f	0.147 ± 0.00 cd	Dense
LSD	**6.2619**	**3.9539**	**0.0543**	

Mean values sharing common letters in each column do not differ significantly at $p<0.05$. Isolates were grown on PDA medium for 7 days. Values are averaged from three replicates. ± showed standard errors values for all the means

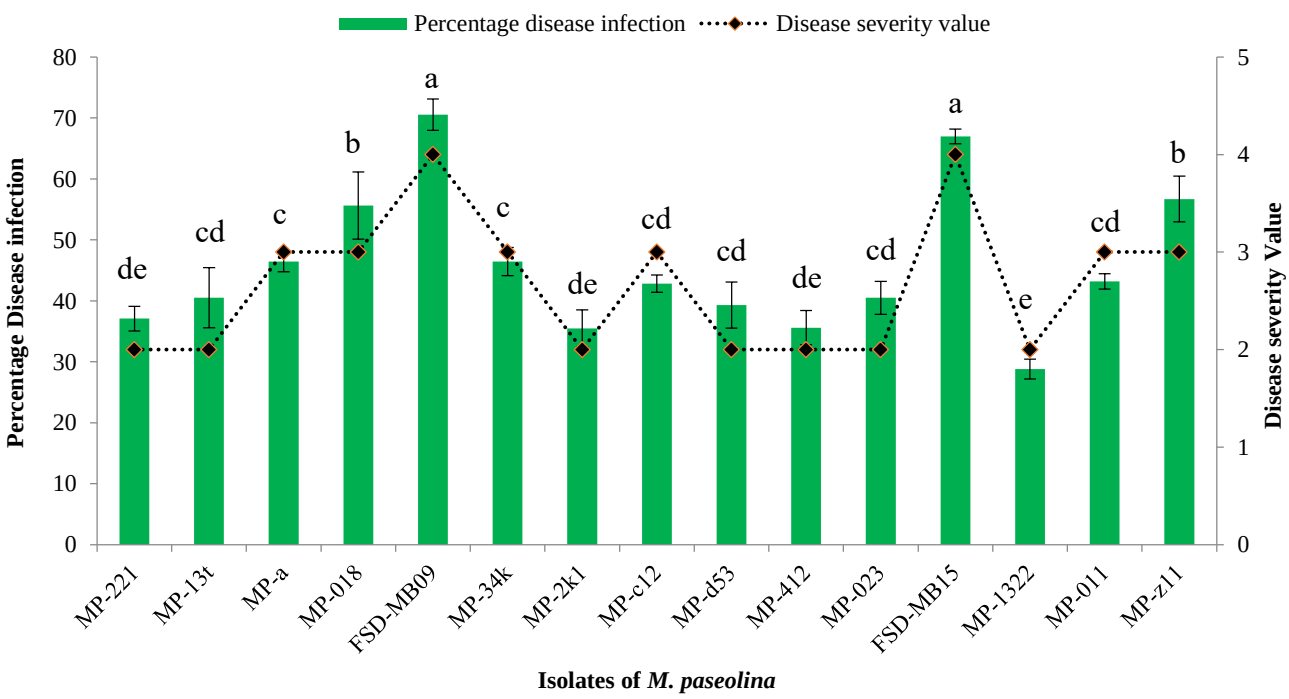

Fig. 2. Pathogenicity assay for the selection of virulent strains of *M. phaseolina*. Pathogenicity assay was performed by placing the surface disinfected mungbean seeds (*cv*; NCM 257-10) on seven days old *M. phaseolina* culture on PDA medium in Petri plates followed by incubation at 26±1°C for twelve days. Chart bars sharing common letters do not differ significantly at p<0.05. Values are averaged from three replicates. Bars showed standard errors values for all the means.

Antagonistic potential of selected fungal agents belonging to *Aspergillus, Trichoderma* and *Penicillum* spp. was tested against the highly virulent strain of *M. phaseolina* (MP-19s) as presented (Table 2 and Fig. 4). All the tested fungal agents successfully inhibited the mycelial growth of *M. phaseolina* to various levels. Mycelial growth of *M. phaseolina* after 120 hours of inoculation (HAI) was ranged from 2.1 ± 0.09 to 4.1 ± 0.35 cm in all the treatments as compared to control where colony growth was recorded 7.8 ± 0.15 cm. Among all test fungal antagonists, *Penicillum* spp. (T1) significantly inhibited the mycelial growth of *M. phaseolina*. Percentage colony growth inhibition in *M. phaseolina* was 71.6%, followed by 64.6% in *A. flavus*, 58% in *Th2* and *Th3* (57.8%) while 54.3% was recorded in *Tv3* as compared to control. Among all the treatments, *A. niger* showed least inhibitory effect (44.5%) on *M. phaseolina*.

Fungal agents with promising antagonistic efficacy in dual culture experiment were further tested for disease suppression and plant growth promotion effects by following seed treatment technique. Data showed variation in disease severity percentage on different cultivars (Table 3). Among all the tested fungal isolates, *T. harzianum* isolate (Th3) showed better efficacy as bio-

control agent. In *T. harzianum* (Th3) treated seeds, disease severity was in range 6.7 to 11.7% on all the mungbean cultivars followed by *Penicillium* spp. 15.0 to 21.7%. Whereas, disease severity percentage in control treatment was ranged from 64.0 to 70.7%. A significant increase in plant growth parameters was observed in all the treatments as compared to control ($p \geq 0.05$). Among all the fungal antagonists, *T. harzianum* (Th3) and *Penicillium* spp. significantly increased the seed germination, root length, plumule length, plant vigor, and biomass in all the tested mungbean cultivars (Tables 4-8). However, *A. flavus* seed treatment was found least effective in disease control and plant growth promotion in both the experiments. Seed treatment with *T. harzianum* (Th3) and *Penicillum* sp. suppressed the disease severity in mungbean up to 50 days over untreated control (Fig. 5).

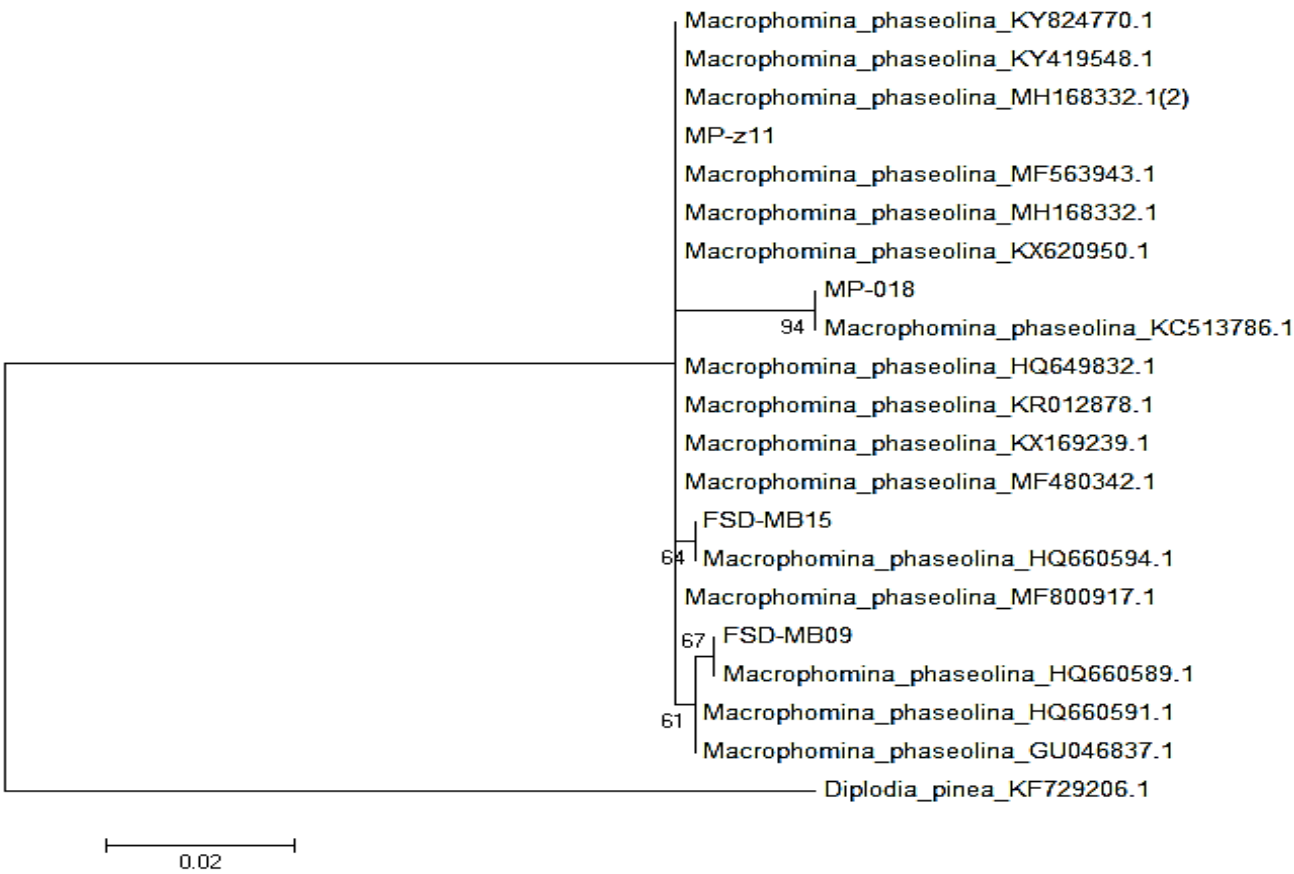

Fig. 3. Phylogenetic analysis of four highly virulent isolates of *M. phaseolina*. Number above the nodes represent bootstrap value >50 after 1000 pseudo replicates. Four isolates of *M. phaseolina* MP-z11, MP-018, FSD-MB15 and FSD-MB09 showed 99-100% sequence homology with GenBank accessions of *M. phaseolina* MH168332, KC513786, HQ660594 and HQ660589 from India and China. All the four sequences were submitted to GenBank under the accession numbers MH371345, MH371315, MH371335 and MH371316.

Table 2. Antagonistic potential of fungal isolates against *M. phaseolina* in vitro.

Fungal strains	Colony growth of *M. phaseolina* (cm)				Percentage colony growth inhibition *in vitro*
	48 HAI	72 HAI	96 HAI	120 HAI	
T1	1.2 ± 0.12 e	1.7 ± 0.38 f	2.0 ± 0.17 f	2.1 ± 0.09 f	71.6%
T2	1.4 ± 0.26 e	2.3 ± 0.23 e	2.5 ± 0.12 e	2.6 ± 0.26 e	64.6%
T3	2.2 ± 0.15 b	3.5 ± 0.58 b	3.9 ± 0.23 b	4.1 ± 0.35 b	44.5%
T4	1.8 ± 0.38 cd	2.9 ± 0.26 cd	3.3 ± 0.43 cd	3.3 ± 0.43 cd	54%
T5	1.7 ± 0.35 d	2.6 ± 0.15 d	2.9 ± 0.38 d	3.1 ± 0.32 d	58%
T6	1.8 ± 0.29 cd	2.7 ± 0.43 cd	3.0 ± 0.48 d	3.1 ± 0.09 d	57.8%
T7	2.0 ± 0.32 bc	3.0 ± 0.58 c	3.5 ± 0.32 c	3.6 ± 0.35 c	52%
T8	1.7 ± 0.17 d	2.7 ± 0.17 d	3.5 ± 0.12 c	3.7 ± 0.20 c	54%
T9	1.9 ± 0.35 cd	3.0 ± 0.32 c	3.0 ± 0.12 d	3.2 ± 0.12 d	54.3%
T10	4.1 ± 0.06 a	5.8 ± 0.15 a	7.1 ± 0.09 a	7.8 ± 0.15 a	0%
LSD	0.2711	0.3613	0.3731	0.4264	

Mean values sharing common letters in each column do not differ significantly at $p<0.05$. Data on fungal growth inhibition was recorded 48, 72, 96 and 120 hours after inoculation (HAI) on PDA medium in vitro. ± represent standard error values. T1 = *Penicillum* spp.; T2 = *Aspergillus flavus*; T3 = *Aspergillus niger*; T4 = *Trichoderma harzianum* (Th1); T5 = Th2; T6 = Th3; T7 = *Trichoderma varidae* (Tv1); T8 = Tv2; T9 = Tv3; T10 = Control

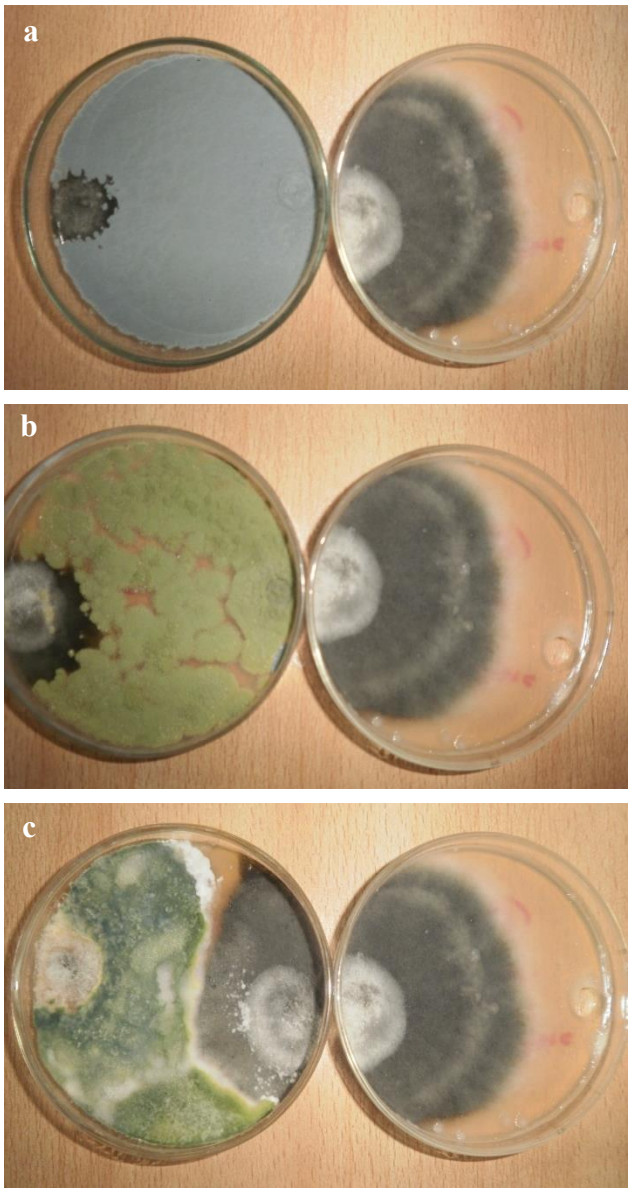

Fig. 4. Antgonistic efficacy of different fungal strains against *M. phaseolina In vitro*; (a) by *Penicillium* spp., (b) *A. flavus* (c) *Trichoderma hazianum* (Th2). Antagonistic ability of tested fungal agents was tested against *M. phaseolina* on PDA medium containing Petri plates.

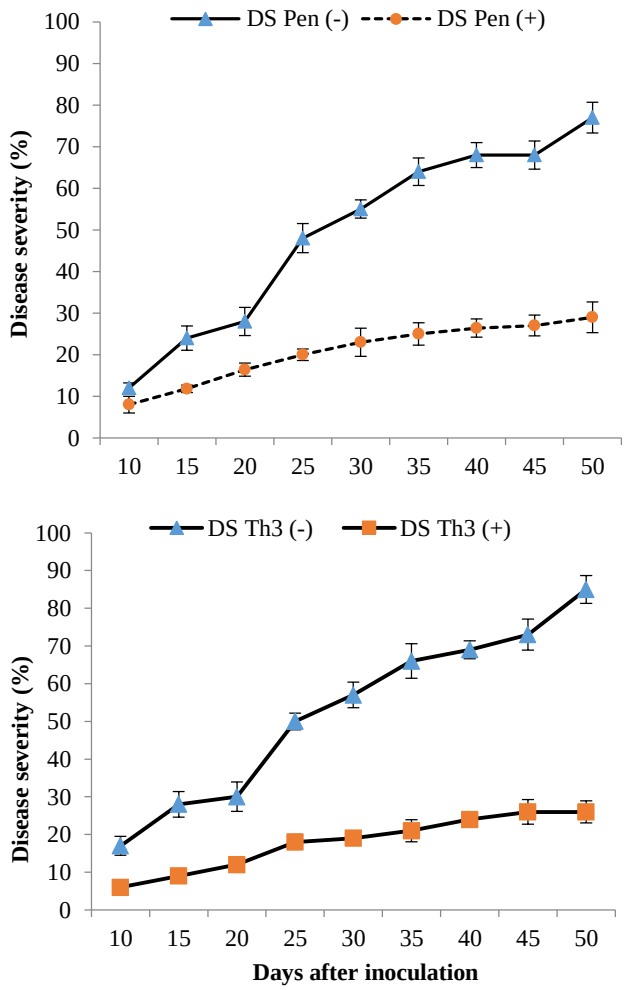

Fig. 5. Disease severity reduction over days in *M. phaseolina* by (a) *Penicillum* spp. and (b) *T. harzianum* (Th3). Bars on each point indicate the standard error for all the means.

Discussion

A total of twelve *M. phaseolina* isolates were recovered from the symptomatic mungbean plant tissues (shoots and roots). Majority of the pathogenic isolates showed similar phenotypic characters with little variability in colony growth rate. A similar research study reported that *M. phaseolina* isolates recovered from various agroecological zones in Pakistan showed variation among the morphological characters (Iqbal & Mukhtar, 2014). Similarly, Riaz *et al.*, (2007) argued that *M. phaseolina* isolates collected from different hosts showed variability. Beas-Fernández *et al.*, (2006) exhibited that *M. phaseolina* isolates recovered from different plant parts also showed variability in phenotypic characters. In our study, *M. phaseolina* was isolated from the infected collar region and roots of the mungbean plants that could be a reason of little variations among the *M. phaseolina* isolates.

Hetrotrogenic nature of *M. phaseolina* makes it diverse in morphology. Pathogenicity plays a significant role in host-plant resistance studies that have been explored by many molecular based studies including Bashasab & Kuruvinashetti (2007); Allaghebandzadeh *et al.*, (2008); Prabhu *et al.*, (2012). In a research study, Purkayastha *et al.*, (2004) found relationship between fungal morphological characters and virulence, while Beas-Fernández *et al.*, (2006); Iqbal and Mukhtar (2014) observed no significant relationship between fungal morphology and pathogenic response.

In molecular assays, ITS regions of rDNA were amplified by using gene specific ITS1 and ITS4 primers. In a broader context, ITS region amplification has become more frequently used approach to identify and differentiate in closely related fungal spp. (Bryan *et al.*, 1995). In this study, four virulent fungal isolates; MP-z11, MP-018, FSD-MB15 and FSD-MB09 were identified as *M. phaseolina*. Accessions MH371345, MH371315, MH371335 and MH371316 showed maximum identity with GenBank accessions of *M. phaseolina* MH168332, KC513786, HQ660594 and HQ660589 respectively. In a similar study, Chakraborty *et al.*, (2011) used the gene specific (ITS1 and ITS2) primers to amplify the ITS regions of rDNA, and

identified *M. phaseolina*. In another study, Živanov *et al.*, (2019) used ITS1 and ITS4 primers and amplified ITS region to identify *M. phaseolina* whereas, Larralde-Corona *et al.*, (2008) used ITS1 and ITS2 primers to identify *M. phaseolina* isolated from sorghum and common bean infected plants.

Disease control mostly relay on fungicide applications, but application of synthetic agro-chemicals pose negative impact on human health, environment and other beneficial microbes. Moreover, chemical based formulations are less economic, and repeated application leads to resistance development in the pathogens (Akhtar and Siddiqui, 2008). Therefore, adaptation of alternative control strategies is required (El-Mougy *et al.*, 2004). Fungal antagonists impede pathogenic activities by competing for space and to meet their nutritional requirements. Moreover, production of specific enzymes/toxins destroy the cell organelles of target pathogens (Agrios, 2005). *Trichoderma* spp. is facultative fungal agents, well-known as biocontrol agents (Druzhinina & Kubicek, 2005). In our studies, *Trichoderma* and *Penicillium* spp. were observed highly emphatic in rebating the *M. phaseolina* on the PDA medium whereas, *A. niger* was found least efficient. *Trichoderma* and *Penicillium* spp. significantly antagonized the *M. phaseolina* over untreated control. Findings of the present studies are in accordance to a research in which *Penicillium* spp. displayed antagonism against *Fusarium* spp. (Alam *et al.*, 2010) and *Claviceps africana* (Bhuiyan *et al.*, 2003). Antagonistic potential of various antagonists has been explored against *M. phaseolina* on Sesame (Sankar & Jeyarajan, 1996), soybean (Vyas, 1994), Pigeon pea (Lokesha & Benagi, 2010), and Eggplant crops (Ramezani, 2008). In another study, cultures of *A. niger*, *Penicillium* spp. and *T. harzianum* inhibited the mycelial growth of *F. oxysporum* f. sp. *lycopersici* in dual culture assays (Alwathnani & Perveen, 2012).

High metabolic rate of the test biocontrol agents, cell wall degrading enzymes, and antibiotics production is an adding factor that enhances the antagonism in biocontrol agents against noxious fungal soil inhibiting pathogens. In a research study, *T. harzianum* and *T. viridae* were reported to be effective fungal antagonists (Dubey & Upadhyay, 2001). In a recent research study, Javeria *et al*., (2020) explored isolates of *T. harzianum* as potential antagonists against *Fusarium oxysporum* f. sp. *lentis* (Fol), and were reported to produce cell wall-degrading enzymes (chitinase and β-1,3 glucanase) along with Indole Acetic Acid (IAA) and Gibberllic Acid (GA3). Production of cell wall-degrading enzymes and plant growth hormones confirm the antagonistic nature of *Trichoderma* spp.

Seed dressing with antagonistic fungal agents increase the seed germination percentage. In many previous studies, *B. thuringiensis*, *R. meliloti*, *A. niger* and *T. harzianum* were studied against *M. phaseolina*, *R. solani* and *Fusarium* spp. In a research study, it was reported that apart from disease reduction, plant parameters were also increased by *Trichoderma* spp. (Dawar *et al.*, 2008). In another study, Bhuiyan *et al.*, (2003) tested *Trichoderma* spp., *Penicillium citrinum*, *Pseudomonas aeruginosa,* and *Burkholderia cepacia* for their biocontrol ability against *Claviceps africana*. Biocontrol potential of fungal agents used in present study was proved by other research works against other plant pathogens such as *Fusarium moniliforme* (Senthil *et al.*, 2011); *C. gloeosporioides* by (Svetlana, *et al*., 2010), and *Fusarium* spp. (Motesharrei & Salimi 2014). Peng & Sutton (1991) demonstrated that 80 – 90% disease severity of the *Botrytis cinerea* was reduced in strawberry by the application of *Penicillium* sp., *T. viride* and *E. nigrum*.

Current study support the idea that fungal microbial agents can be a potential alternative to synthetic agrochemicals in disease management programs. Up till now, no fungal based bio-formulation is available in Pakistan. Present study can be helpful to focus on developing and testing bio-fungicide under field conditions. In depth study is required to explore the biocontrol potential of *Trichoderma* and *Penicillium* spp., which may lead to the development of economical bio-pesticide to control soil-borne pathogens, like many other fungal based bio-formulations are available as myco-fungicides (Kaewchai *et al.*, 2009). Beside these evidences, antifungal products can be extracted and characterized, which may be beneficial as potential antagonist against many other fungal pathogens.

Table 3. Effect of various fungal strains on disease severity reduction against *M. phaseolina* on mungbean.

Fungal isolate	Mean disease severity percentage				
	NM-98	AUM-19	AUM-38	AUM-6173	MG-1
T1	21.7 hij	20.0 ijk	15.0 klmno	18.3 ijkl	16.7 jklmn
T2	38.3 de	45.0 c	33.3 ef	41.7 cd	35.0 e
T3	13.3 lmno	18.3 ijkl	23.3 ghi	20.0 ijk	21.7 hij
T4	11.7 mnop	10.7 nop	9.0 op	11.7 mnop	6.7 p
T5	28.5 fg	26.7 gh	16.7 jklmn	17.3 ijklm	16.7 jklmn
T6	70.7 a	70.0 ab	64.0 b	64.7 ab	65.0 ab
LSD	6.4922				

*Values are means of two experiments with five replicates. Values sharing common letters in each column do not differ significantly at p≤0.05 according to least significant difference test; all values are significantly lower than that of the control treatment. T1 = *Penicillum* spp.; T2 = *Aspergillus flavus*; T3 = *T. harzianum* (Th2); T4 = *T. harzianum* (Th3); T5 = *T. viridae* (Tv3); T6 = control

Table 4. Effect of fungal antagonists on seed germination percentage and plant growth promotion on NM-98.

Fungal strains	NM-98									
	Experiment 1					Experiment 2				
	GP %	RL (cm)	PL (cm)	VI	BM (g)	GP %	RL (cm)	PL (cm)	VI	BM (g)
T1	91.4	14.97 a	6.73 a	1983.4 a	3.37 a	90.0	15.30 a	7.23 ab	2029.2 a	3.30 ab
T2	75.4	13.17 b	5.87 ab	1435.1 c	3.23 a	81.7	11.23 c	5.50 bc	1363.8 b	2.33 b
T3	78	12.16 bc	6.0 ab	1417.0 c	2.70 ab	70.0	13.10 b	6.23 bc	1353.3 b	2.90 ab
T4	87	13.1 b	6.10 ab	1667.5 b	2.77 ab	93.0	14.87 a	8.33 a	2161.0 a	3.57 a
T5	73.4	11.10 c	5.4 b	1211.1 d	2.20 bc	75.7	12.57 b	5.37 c	1357.5 b	2.50 ab
T6	53.4	6.73 d	4.30 c	589.18 e	1.53 c	49.0	5.33 d	4.17 d	465.0 c	0.90 c
LSD	-	1.4736	1.0441	151.06	0.8098	-	1.2794	1.2904	235.17	1.1126

Mungbean variety NM-98 was used in this repeated experiment with five replications for each treatment. T1 = *Penicillum* spp.; T2 = *Aspergillus flavus*; T3 = *T. harzianum* (Th2); T4 = *T. harzianum* (Th3); T5 = *T. viridae* (Tv3); T6 = control. Data on Germination percentage (GP); Radical length (RL); Plumule length (PL); Vigor index (VI); Biomass (BM) was recorded. Mean values sharing common letters in each column do not differ significantly at p≤0.05 according to least significant difference test

Table 5. Effect of fungal antagonists on seed germination percentage and plant growth promotion on AUM-19.

Fungal strains	AUM-19									
	Experiment 1					Experiment 2				
	GP %	RL (cm)	PL (cm)	VI	BM (g)	GP %	RL (cm)	PL (cm)	VI	BM (g)
T1	92.3	15.20 a	7.37 a	2083.1 a	3.23 a	85.0	14.0 ab	7.77 a	1845.5 ab	2.80 ab
T2	84.0	13.60 abc	5.53c	1607.9 bc	2.73 ab	76.3	10.17 d	4.90 bc	1146.3 c	2.37 b
T3	86.3	13.17 bc	6.77 ab	1721.3 bc	3.10 a	85.0	12.77 bc	6.37 ab	1630.0 b	2.67 b
T4	89.3	14.27 ab	6.13 bc	1820.1 b	2.27 bc	92.7	14.87 a	7.30 a	2052.6 a	3.27 a
T5	85.7	12.03 c	5.63 c	1509.7 c	2.40 bc	77.0	11.37 cd	6.13 ab	1342.4 c	2.07 bc
T6	61.0	5.80 d	4.17 d	615.23 d	1.77 c	55.0	4.63 e	3.70 c	454.0 d	1.20 c
LSD	-	1.6142	0.8925	220.34	0.6709	-	2.0266	1.6657	263.09	0.8993

Mungbean variety AUM-19 was used in this repeated experiment with five replications for each treatment. T1 = *Penicillum* spp.; T2 = *Aspergillus flavus*; T3 = *T. harzianum* (Th2); T4 = *T. harzianum* (Th3); T5 = *T. viridae* (Tv3); T6 = Control. Data on Germination percentage (GP); Radical length (RL); Plumule length (PL); Vigor index (VI); Biomass (BM) was recorded. Mean values sharing common letters in each column do not differ significantly at $p \leq 0.05$ according to least significant difference test

Table 6. Effect of fungal antagonists on seed germination percentage and plant growth promotion on AUM-38.

Fungal strains	AUM-38									
	Experiment 1					Experiment 2				
	GP %	RL (cm)	PL (cm)	VI	BM (g)	GP %	RL (cm)	PL (cm)	VI	BM (g)
T1	88.3	14.83 a	8.50 a	2060.7 a	3.27 a	85.0	13.37 a	8.90 a	1892.7 a	3.20 a
T2	85.0	12.07 bc	5.73 c	1518.5 b	2.50 b	83.3	11.10 b	6.20 b	1442.3 b	2.23 c
T3	80.7	11.97 bc	6.57 bc	1492.8 b	2.93 ab	81.7	11.63 b	7.10 b	1527.8 b	2.97 ab
T4	90.0	13.57 ab	7.73 ab	1920.3 a	3.33 a	95.0	12.17 b	8.27 a	1941.2 a	3.53 a
T5	91.7	10.57 c	6.00 c	1519.7 b	3.07 ab	78.3	9.63 c	6.50 b	1264.7 c	2.50 bc
T6	68.3	6.50 d	3.63 d	690.17 c	1.57 c	56.7	5.90 d	4.20 c	570.67 d	1.20 d
LSD	-	2.2228	1.4052	274.40	0.6191	-	1.1070	0.9672	149.33	0.6033

Mungbean variety AUM-38 was used in this repeated experiment with five replications for each treatment. T1 = *Penicillum* spp.; T2 = *Aspergillus flavus*; T3 = *T. harzianum* (Th2); T4 = *T. harzianum* (Th3); T5 = *T. viridae* (Tv3); T6 = Control. Data on Germination percentage (GP); Radical length (RL); Plumule length (PL); Vigor index (VI); Biomass (BM) was recorded. Mean values sharing common letters in each column do not differ significantly at $p \leq 0.05$ according to least significant difference test

Table 7. Effect of fungal antagonists on seed germination percentage and plant growth promotion on AUM-6173.

Fungal strains	AUM-6173									
	Experiment 1					Experiment 2				
	GP %	RL (cm)	PL (cm)	VI	BM (g)	GP %	RL (cm)	PL (cm)	VI	BM (g)
T1	93.3	12.77 a	8.13 a	1947.7 a	2.97 ab	90.0	12.30 ab	7.30 ab	1762.0 b	3.10 ab
T2	86.7	10.23 bc	5.33 c	1349.0 cd	2.50 ab	83.3	10.37 c	5.37 d	1311.2 d	2.20 c
T3	91.7	11.57 ab	6.23 bc	1631.0 bc	2.20 b	87.0	11.3 bc	6.23 cd	1528.2 c	2.87 ab
T4	95.0	12.60 ab	7.43 ab	1903.2 ab	3.20 a	96.3	13.20 a	7.77 a	2010.3 a	3.43 a
T5	88.3	9.03 cd	5.43 c	1283.7 d	2.77 ab	80.0	10.87 c	6.77 bc	1402.7 cd	2.47 bc
T6	73.3	6.57 d	3.73 d	759.83 e	1.17 c	60.0	5.17 d	4.33 e	570.17 e	0.77 d
LSD	-	2.5312	1.3173	292.52	0.9985	-	1.0533	0.8826	156.87	0.6387

Mungbean variety AUM-6173 was used in this repeated experiment with five replications for each treatment. T1 = *Penicillum* spp.; T2 = *Aspergillus flavus*; T3 = *T. harzianum* (Th2); T4 = *T. harzianum* (Th3); T5 = *T. viridae* (Tv3); T6 = control. Data on Germination percentage (GP); Radical length (RL); Plumule length (PL); Vigor index (VI); Biomass (BM) was recorded. Mean values sharing common letters in each column do not differ significantly at p≤0.05 according to least significant difference test.

Table 8. Effect of fungal antagonists on seed germination percentage and plant growth promotion on MG-1.

Fungal strains	MG-1									
	Experiment 1					Experiment 2				
	GP %	RL (cm)	PL (cm)	VI	BM (g)	GP %	RL (cm)	PL (cm)	VI	BM (g)
T1	91.7	10.77 bc	8.00 a	1720.7 a	2.03 b	97.0	11.37 ab	8.20 ab	1897.6 a	2.70 bc
T2	85.0	8.73 d	6.03 bc	1255.2 bc	2.43 ab	80.0	7.87 c	6.23 d	1135.5 c	2.03 d
T3	81.7	13.23 a	5.73 bc	1552.2 ab	2.87 ab	90.0	10.93 ab	7.87 bc	1692.0 b	2.50 cd
T4	94.0	11.60 ab	7.40 ab	1788.7 a	3.27 a	95.0	12.30 a	8.77 a	1991.8 a	3.50 a
T5	80.0	9.43 cd	5.07 cd	1163.8 c	2.67 ab	85.0	10.23 b	7.33 c	1493.2 b	3.13 ab
T6	68.3	5.60 e	3.40 d	613.33 d	0.97 c	60.0	6.10 d	4.23 e	621.67 d	0.83 e
LSD	-	2.0079	1.6830	306.83	1.0525	-	1.3901	0.5563	200.14	0.5435

Mungbean variety MG-1 was used in this repeated experiment with five replications for each treatment. T1 = *Penicillum* spp.; T2 = *Aspergillus flavus*; T3 = *T. harzianum* (Th2); T4 = *T. harzianum* (Th3); T5 = *T. viridae* (Tv3); T6 = control. Data on Germination percentage (GP); Radical length (RL); Plumule length (PL); Vigor index (VI); Biomass (BM) was recorded. Mean values sharing common letters in each column do not differ significantly at $p \leq 0.05$ according to least significant difference test

Conclusion

Results from the present work displayed antagonist potential of selected fungal antagonistic agents against highly virulent strain of *M. phaseolina* infecting mungbean crop. Among all the tested fungal isolates, *Penicillium* spp. and *Trichoderma harzianum* significantly suppressed mycelial growth of *M. phaseolina*, reduced disease severity, enhanced percentage seed germination, and plant growth promotion under net house conditions.

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