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Integrated management of charcoal rot disease of soybean caused by *Macrophomina phaseolina*

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ABSTRACT

An attempt was taken for the management of charcoal rot disease of soybean caused by *Macrophomina phaseolina* using the integration of bio-agent with fungicide and organic amendment. Before going to the field experiments, different *in-vitro* trials were conducted to select a virulent isolate of *M. phaseolina*, an effective antagonistic isolate of *Trichoderma harzianum,* suitable fungicide and organic amendment. Among the seven isolates of *M. phaseolina*, MSP-4 isolate was selected as the test pathogen by the pathogenicity test. On the contrary, among the eighty-seven isolates of *T. harzianum*, ISR-26 isolate showed the highest (81.85%) inhibition of radial growth of test pathogen. In the case of fungicidal evaluation trial, Provax 200WP was found the most effective seed treating fungicide at the moderate concentration (150 ppm), while experienced the highest concentration of Conza 5%EC and Bavistin 50WP for inhibiting the radial growth of *M. phaseolina* isolate MSP-4. Additionally, *in-vitro* trial of different organic amendments, mustard oil cake was found the most active organic amendment for reducing the growth (50.92%) and development of test pathogen at 3% concentration level. In the field trial, integrated use of *T. harzianum* with Provax 200WP, and mustard oil cake under the treatment with T_9 appeared the best treatment in reducing seedling mortality (80.47%), disease incidence (80.14%) as well as disease severity (88.55%) caused by the test pathogen. Moreover, treatment T_9 was the best treatment not only for the management of the soybean disease but also increased the significant quantity of yield (104.42%). This integrated approach could be more helpful for soybean growers all over the world.
Keywords: *M. phaseolina*, *T. harzianum*, Fungicides, Organic amendments, and Soybean

INTRODUCTION

Soybean (*Glycine max* L.) belongs to the family Leguminosae which contributes around twenty-five percent of the global edible oil as well as about two-thirds of the world's protein concentrate for livestock feeding. Additionally, soybean meal is a valuable constituent in formulated feeds for poultry and fish. In 2017-18, the total soybean cultivable land was 59.49 thousand hectares and yield production was 99 thousand tons with an average yield of 1.66 t/ha (Anon, 2018). Nowadays, soybean is becoming a popular winter crop in Bangladesh. Growers are becoming interested to cultivate soybean instead of other legume crops. But there are some challenges for the production of soybean such as quality seeds, climatic conditions, differences in rainfall pattern, an outbreak of diseases and pests, etc. Among these features, plant diseases are one of the most important factors for the cultivation of soybean. More than hundreds of plant pathogens are identified to affect soybean where sixty-six fungi, six bacteria, eight viruses and seven nematodes (Sinclair, 1988).

Macrophomina phaseolina is one of the most destructive pathogens which can be spread by contaminated seeds and soil (Yesil and Bastas, 2016). This notorious soil-borne fungus causes charcoal rot disease of soybean. The symptoms of charcoal rot disease generally occur during the seedlings to flowering stage. The major characteristic is to develop brown lesions on the hypocotyl of emerging seedlings. Amid growing season, leaflets on infected plants may be small, wither and turn a brown color. The taproot of the plant and ground line stem may become streaked with a light grey color. A plethora of small black specks may form underneath of epidermis and inside the lower stem and taproot which appears charcoal-sprinkled. Finally, the reddish-brown discoloration can also develop in the pith and vascular tissues of the root and the plant become wilted and die.

However, management of this pathogen is sometimes difficult because of its long persistence in seed and wide host range. Chemical fungicides are effective against this fungus but these chemicals are expensive and harmful for humans and animals. Not only detrimental for living organisms but also contaminated the environment rigorously. Moreover, indiscriminate use of chemical pesticides and fertilizers in modern agriculture has resulted in the development of several problems such as pesticide resistance in pests, the resurgence of target and non-target pests, destruction of beneficial organisms like honey bees, and chemical residues in food, feed and fodder (Arefin *et al.,* 2019). On the other hand, Integrated Disease Management (IDM) strategy is comparatively safe, environment friendly and durable. Integration of chemical, cultural and biological approaches to control *M. phaseolina* may be most effective rather than discretely application (Abd-Elsalam, 2010). Effective and proficient use of chemicals, bio-control agents and organic amendments, therefore, may be potential to control the charcoal rot disease of soybean caused by *M. phaseolina*. Several reporters (Rubayet and Bhuiyan, 2016; Liton *et al*., 2019; Ahmed *et al.,* 2019) found that combined application of fungicide, organic amendment (mustard oilcake), biocontrol (*Trichoderma* sp.) is highly effective for controlling the soil-borne as well as seed-borne plant pathogens during crops cultivation (Aly *et al.,* 2007). This combined package not only minimizes plant diseases but also improves soil health and ultimately crop production. However, a few studies have been done on the management of charcoal rot disease of soybean but there is no report on integrated management of the above-mentioned disease of soybean in Bangladesh.

Considering the aforesaid facts, the present research was undertaken to evaluate the effectiveness of integrated disease management strategies consisted of bio-agent, fungicide and organic amendment against charcoal rot disease of soybean caused by *M. phaseolina*.

MATERIALS AND METHODS:

Isolation and preservation of *M. phaseolina* isolates:

Seven isolates of *M. phaseolina* designated as MSP-1 to MSP-7were isolated from infected root, and pod tissues of soybean, bush bean, and pea. The specimens which had typical symptoms of root rot were selected from the infected fields. The fungal isolates were isolated according to the standard method (Mian, 1995). Then, the fungal colonies were grown on PDA and identified according to Barnett and Hunter (1972). The isolates were purified following the hyphal tip technique and stored in PDA slants at 10°C.

Cultural characterization of *M. phaseolina* isolates:

The selected isolates MSP-1 to MSP-7 were individually inoculated into three replicated PDA plates and incubated according to the Rahman *et al.* (2020b). After 7 days of incubation, observation on cultural characteristics such as colony color, colony type, pycnidia population, and pycnidia distribution was recorded. The isolates colony type and colony color were fluffy and dark grey. The pycnidia and pycnidiospores were observed under a compound microscope.

Inoculum preparation of the tested pathogen:

Inoculum of the test pathogen isolates was made and kept following the standard method (Rubayet and Bhuiyan, 2016).

Pathogenicity test:

The pathogenicity test was done of seven selected isolates of *M. phaseolina* designated as MPS-1 to MPS-7 against soybean seedlings in the pot culture experiment according to the standard methods (Rubayet *et al.,* 2017; Liton *et al.,* 2019).

Collection, isolation and preservation of *T. harzianum* isolates:

A total of 87 isolates of *T. harzianum* whereas 37 isolates were isolated from the different crop fields of Gazipur, Chuadanga and Meherpur districts of Bangladesh following the soil dilution plate technique (Dhingra and Sinclair, 1985). And rest of the 50 isolates were collected directly from the plant pathology laboratory, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Bangladesh. All the isolated *Trichoderma* spp. were identified as *T. harzianum* based on the different morphological characteristics like hyphal growth, spore formation and color. The pure culture of *T. harzianum* was conserved for future application (Das et al., 2019).

Screening of *T. harzianum* against *M. phaseolina* isolate MSP-4:

The *in-vitro* screening was accompanied to assess the antagonistic effect of selected 87 isolates of *T. harzianum* against test pathogen on PDA medium by dual plate culture technique (Dhingra and Sinclair, 1985). After 7 days of incubation, the inhibition percentage of radial growth of the test pathogen was calculated using the following formula (Sundar *et al.,* 1995).

$$\%\ \text{inhibition of growth} = \frac{P - Q}{P} \times 100$$

Where, P = Mycelial growth of the pathogen in absence of *T. harzianum* (control), and Q = Mycelial growth of the pathogen in presence of *T. harzianum*.

Evaluation of fungicides and organic amendments against *M. phaseolina* isolate MSP-4:

Effect on radial colony growth:

Five fungicides (Conza 5%EC, Cabrio*Top 60WP, Provax 200WP, Bavistin 50WP and Dithane M-45) at three different concentrations viz., 75, 150, and 300 ppm were assessed their effect on radial colony growth following poison food technique (Dhingra and Sinclair, 1985) (**Table 1**). Three replicated plates with CRD were used for each dose of each fungicide. The inoculated plates were incubated in the laboratory having an ambient temperature of 28±3 °C (Rubayet *et al.,* 2011). Data on radial colony diameter was recorded after 3-days of incubation when the check plate was enclosed with the growth of the test pathogen. The diameter of colonies on PDA with and without fungicide was measured from the bottom side of the Petri dishes. The inhibition of radial colony growth in amended plates was calculated based on colony diameter of the check plate following the formula as suggested by Sundar et al. (1995) mentioned earlier.

Another *in-vitro* experiment was conducted to determine the effect of organic amendments (mustard oil cake, sesame oil cake, soybean oil cake, coconut oil cake, and tea waste) at 3 different concentrations viz. 1, 2, and 3% on the growth of *M. phaseolina* isolate MSP-4 following standard techniques (Dhingra and Sinclair, 1985; Rubayet *et al.,* 2011; Rubayet *et al.,* 2018). Three days after incubation the inhibition of radial colony growth in the amended plates was computed based on colony diameter of the control plate using the same formula as stated above by Sundar *et al.* (1995).

Table 1. List of fungicides and their active ingredients applied in the current study

Fungicides	Active ingredients	Modes of action
Conza 5%EC	Hexaconazole 5% EC	Systemic
Cabrio*Top	Pyroclostribin 5% + Metirum 55% WP	Systemic and contact
Provax 200WP	Carboxin 37.5% + Thiram 37.5% WP	Systemic and contact
Bavistin 50WP	Carbendazim 50% WP	Systemic
Dithane M-45	Mancozeb 80% WP	Systemic and contact

Effect on mycelial dry weight:

The effect of the mentioned fungicides on mycelial dry weight of *M. phaseolina* isolate MSP-4 was determined by Rahman et al. (2020a). In the meantime, the effect of organic amendments on mycelial dry weight of *M. phaseolina* isolate MSP-4 was determined by growing fungi in the Potato Dextrose Broth (PDB) amended with individual organic amendments at the concentration of 1, 2, and 3% (v/v) following the same technique as described earlier (Dhingra and Sinclair, 1985). Inhibition of mycelial dry weight in the amended broth was calculated based on the dry weight in control treatment following the above-mentioned formula.

Compatibility of *T. harzianum* isolate ISR-26 with fungicides and organic amendments:
The compatibility test of *T. harzianum* isolate ISR-26 with fungicides and organic amendments were justified according to Rubayet and Bhuiyan (2012), and Rahman *et al.* (2020a).

Preparation of inoculum:
The selected isolate of *M. phaseolina* isolate MSP-4 and *T. harzianum* isolate ISR-26 were used for the production of wheat grain colonized inoculum distinctly following the standard procedures (Rubayet and Bhuiyan, 2016).

Integrated effect of *Trichoderma*, fungicide and organic amendment on *M. phaseolina* isolate MSP-4:
A field experiment was conducted to find out the effect of integrated use of *T. harzianum* isolate ISR-26, seed treating fungicide Provax-200 WP, and mustard oil cake against the charcoal rot disease of soybean caused by *M. phaseolina* isolate MSP-4 and response on yield production. The test pathogen was artificially inoculated in the respective experimental field before sowing the seeds.

Cultivation of soybean:
Cultivable land was prepared and made the plot according to Rahman *et al.* (2018). Nine different treatments were allotted randomly to nine-unit plots per block. Before sowing, seeds were soaked for 24 hours to facilitate the germination and also dried for avoiding excess water. For the respective treatment of trial, seeds were treated with Provax 200 WP @ 0.2 g/100 g seeds. Then, seeds were sown in lines uniformly by hand (45 Kg/ha) keeping the row-to-row distance of 25 cm. Weeding, mulching and irrigation were done in the experimental field whenever necessary.

Treatments of the field experiment:
T_1= Fresh seeds sown in sterilized (5% formaldehyde) soil (Control-1), T_2= Soil inoculated with pathogen (SIP) + Fresh seeds (Control-2), T_3= SIP + Fungicide Treated Seeds (FTS), T_4= SIP + Wheat Grains Colonized *T. harzianum* isolate ISR-26 (WGCT) + Fresh seeds, T_5= SIP+ Organic Amendment (OA) + Fresh seeds, T_6= SIP+ WGCT + FTS, T_7= SIP+ WGCT + OA + Fresh seeds, T_8= SIP+ OA + FTS, and T_9= SIP+ WGCT + OA + FTS.

Treatments application methods:
Nine treatments were tested in the open field under artificially inoculated conditions. Control-1 was sterilized with 1% formaldehyde by drenching the soil properly. After treatment with formaldehyde, the soil was covered with transparent polyethylene sheets. Polyethylene sheets were removed after 48 hours and exposed to air 7 days before sowing. Inoculum of a selected isolate of *M. phaseolina* was thoroughly mixed with soil according to design and layout @ 90 g/m^2 soil as suggested by Yuen *et al.* (1994). Water-soaked sterilized and air-dried wheat grains but not colonized by the fungal isolate was inoculated at the same rate in the control plots. Mustard oil cake was mixed with the soil of concerning treatments plot was used @ 5 t/ha. After 21-days, wheat grains colonized *T. harzianum* isolate ISR-26 was mixed thoroughly with the soil of selected treatments @ 50 g/m^2 (Abd-El-Khair *et al*., 2010). Then after three days, soybean seeds were sown in the plots of all treatments. In the case of seeds treatment with fungicide, around 100 g seeds were taken in a conical flask then added 0.2 g Provax 200WP and mixed properly before sowing.

Data recording:
The number of emerged seedlings was recorded after 15-days of sowing and converted into percent of pre-and post-emergence mortality in seedlings. Diseased seedlings were counted every alternate day and continued up to 30 days after sowing (Rahman *et al*., 2018). Germination and seedling mortality were expressed in percentage based on the total number of seeds planted. The disease incidence (DI), percent disease index (PDI) and total yield were assessed by the following formulas (Rahman *et al.,* 2013; Razaq *et al.,* 2015).

$$\text{DI} = \frac{\text{Number of infected plants}}{\text{Total number of plants assessed}} \times 100$$

$$\text{PDI} = \frac{\text{Summation of all ratings}}{\text{Total number of rating} \times \text{Max. disease grade (4)}} \times 100$$

$$\text{Total pod yield (t/ha)} = \frac{\text{Yield per plot (kg)}}{\text{Area of plot (m}^2\text{)} \times 1000\ \text{(kg)}} \times 10000\ \text{m}^2$$

Data analysis:
Statistically, data were analyzed using the MSTAT-C computer program after proper transformation whenever it was necessary. The treatment means were compared following Duncan's Multiple Range Test (DMRT).

RESULTS

Isolation and cultural characterization of *M. phaseolina* isolate MSP-4:
All the isolates were identified based on the morphological characteristics of pycnidia, pyncidiospores, conidiophores, etc. which were produced on PDA medium (Wheeler, 1975). Pycnidia were 100-200 µm in diameter, dark to greyish, becoming black with age, globose or flattened globose, membranous to sub-carbonaceous with rod-shaped conidiophores and shorten ostiole. Microsclerotia were developed by hyphal cell aggregation. It was black and are variable in size (50–150 µm) (**Table 2 and Fig. 1**).

Table 2. Cultural characteristics of *M. phaseolina* isolates on PDA medium

Isolates	Locations	Source Crops	Colony colors	Colony types
MPS-1	BSMRAU	Jute	Dark Gray	Fluffy
MPS-2	Meherpur	Soybean	Dark Gray	Fluffy
MPS-3	BSMRAU	Soybean	Dark Gray	Fluffy
MPS-4	BSMRAU	Soybean	Dark Gray	Fluffy
MPS-5	BSMRAU	Papaya	Dark Gray	Fluffy
MPS-6	BSMRAU	Eggplant	Dark Gray	Fluffy
MPS-7	Chuadanga	Soybean	Dark Gray	Fluffy

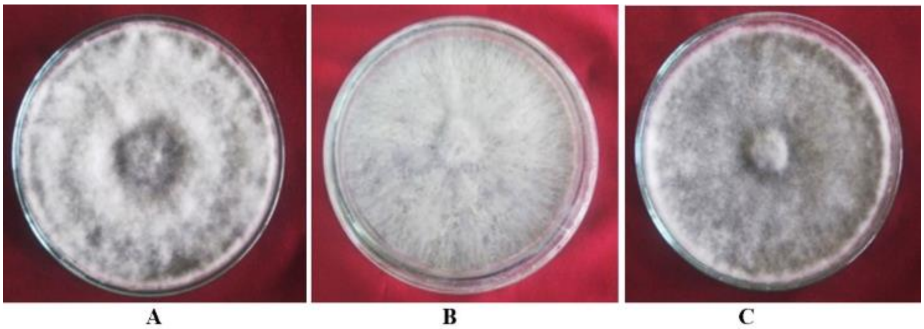

Fig. 1. Cultural variation of *M. phaseolina* isolates on PDA medium (A-C).

Pathogenicity test of *M. phaseolina* isolates in pot culture:

All the isolates of *M. phaseolina* showed variability in their pathogenicity causing 29.62 to 74.06% seedling mortality due to pre-and post-emergence damping-off of soybean. The isolate MPS-4 showed the highest pre-emergence seedling mortality 48.14%, followed by MPS-3 (40.72%). Additionally, the highest post-emergence seedling mortality was found in isolate MPS-4 (25.92%) followed by MPS-2 (18.52%). Moreover, the total mortality (74.06%) was observed in MSP-4 isolate (**Table 3**). Based on the present findings isolate MPS-4 was selected for further study.

Table 3. Pathogenicity test of *M. phaseolina* isolates in pot culture

Isolates	% seedling mortality		
	Pre-emergence	**Post-emergence**	**Total**
MPS-1	25.92	11.11	37.03 $^{c\delta}_{(37.44)}$ ^{ß}
MPS-2	18.52	18.51	37.03 $^{c}_{(37.44)}$
MPS-3	40.72	14.81	55.55 $^{b}_{(48.19)}$
MPS-4	48.14	25.92	74.06 $^{a}_{(59.99)}$
MPS-5	14.81	14.81	29.62 $^{c}_{(32.88)}$
MPS-6	29.63	7.40	37.03 $^{c}_{(37.44)}$
MPS-7	37.03	3.70	40.73 $^{c}_{(39.63)}$
Control	0.00	0.00	0.00 $^{d}_{(1.654)}$

^{ß} Figures within the parentheses are the transformed (arcsine) values. $^{\delta}$ Means within same column followed by a common letter(s) are not significantly different (α = 0.05).

Screening of *T. harzianum* against *M. phaseolina* Isolate MSP-4:

An *in-vitro* experiment was conducted to evaluate the antagonistic efficiency of 87 isolates of *T. harzianum* against one selected isolate of *M. phaseolina isolate* MSP-4 following the dual plate culture technique. The highest 81.85% reduction of mycelial growth of test pathogen was found with the *T. harzianum* isolate ISR-26 followed by the isolate MYT-75 (80.74%), and DT-5 (80.19%) (Fig. 2 & 3). On the other hand, the lowest inhibition (55.74%) in radial growth was observed with the isolate THC-6 against the test pathogen. Moreover, all *T. harzianum* showed a great variation in their degree of antagonism which differs from isolate to isolate against test pathogen. The degree of antagonism as measured based on the class number on the indexing scale of 1-5 ranged from 1 to 3 against *M. phaseolina* isolate MSP-4. A total of 15 isolates (17.24%) showed antagonism class at 1, 31 isolates (35.63%) class at 2, 41 isolates (47.13%) class at 3, and no isolate was recorded under the antagonism class at 4 or 5 (**Table 4**).

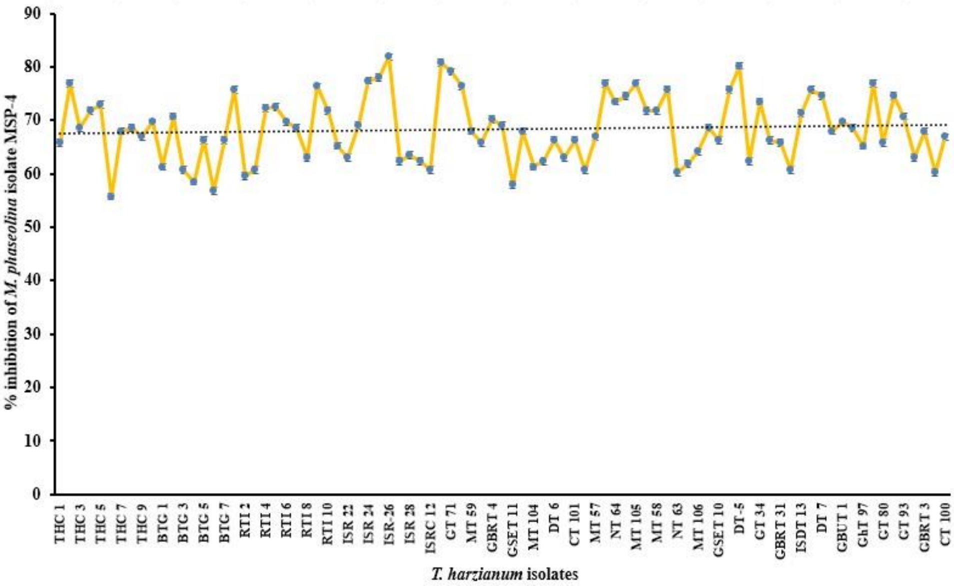

Fig. 2. Percent inhibition of *M. phaseolina* isolate MSP-4 mycelial growth by *T. harzianum* isolates in dual culture on PDA.

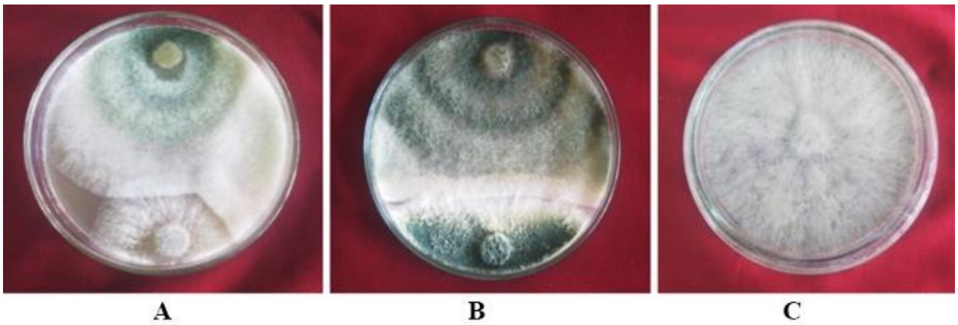

Fig. 3. Antagonism of *T. harzianum* against *M. phaseolina* isolate MSP-4 on PDA (A = ISR-26, B = DT-5, C= Control).

Table 4. Antagonism of *T. harzianum* isolates against *M. phaseolina* isolate MSP-4 in dual culture on PDA

Classes	***T. harzianum* isolates**	Number of isolates	% isolates
1	ISR-26, DT-5, MYT-75, GT-71, ISR-25, ISR-24, MT-105, GT-76, THC-2, GT-36, RTI-9, MT-55, RTI-1, CT-99, CT-102	15	17.24
2	RTI-4, NT-66, GT-44, DT-7, GT-34, NT-64, THC-5, RTI-5, MT-107, RTI-10, MT-58, THC-4, ISDT-13, BTG-2, GT-93, GBRT-4, THC-10, GBUT-1, RTI-6, GT-35, ISR-23, GT-23, RTI-7, MT-52, THC-8, THC-3, GUT-19, THC-7, MT -59, MT-51, GBRT-3	31	35.63
3	ISDT-15, NT-65, TT-112, RTI-8, MT-53, BTG-7, MT-104, THC-9, THC-1, GBRT-31, GT-74, CT-100, MT-57, MT-106, GT-80, DT-6, RT-90, ISR-28, GBUT-2, CT-101, GHT-98, GSET-10, ISDT-16, ISR-22, GT-77, ISR-27, GHT-97, BTG-5, ISR-21, ISRC-12, NT-63, BTG-3, ISRC-11, BTG-1, RTI-3, GT-20, RTI-2, BTG-4, GSET-11, BTG-6, THC-6	41	47.13
4	_	_	_
5	_	_	_
Total		87	

Efficacy of fungicides against *M. phaseolina* isolate MSP-4:

In-vitro trial, the fungicides such as Provax 200WP and Conza 5%EC at higher concentrations (150 ppm and 300 ppm) and Bavistin 50WP at 300 ppm were completely inhibited the radial growth of *M. phaseolina*. The second-best result was obtained with Bavistin 150WP ppm followed by 75 ppm. Cabrio*^{Top} showed reverse result against mycelial growth of the test pathogen even at the highest concentration. Besides, Dithane M-45 showed a moderate inhibitory effect on radial growth which was 28.88, 42.59, and 65.55% at 75 ppm, 150 ppm, and 300 ppm, respectively (**Table 5 and Fig. 4**).

Similarly, the result of the inhibition of mycelial dry weight was also found alike with the five fungicides. The maximum inhibition of mycelial dry weight was achieved with all concentrations of Bavistin 50WP and with 150 ppm and 300 ppm of Provax 200WP and Conza 5%EC, respectively. Conza 5%EC at 75 ppm showed 87.64% inhibition followed by Provax 200WP (82.63%) at 75 ppm, and Dithane M-45 (80.96%) at 300 ppm, respectively. Cabrio*^{Top} showed very poor performance in inhibiting mycelial dry weight. The results of the present study revealed that Conza 5%EC was the most effective fungicide against *M. phaseolina* isolate MSP-4 which was followed by Provax 200WP, and Bavistin 50WP, respectively.

Table 5. *In-vitro* evaluation of fungicides against the radial growth and mycelial dry weight of test pathogen

Fungicides	**Concentrations (ppm)**	**% Inhibition in *M. phaseolina* isolate MSP-4**	
		Radial growth	**Mycelial dry weight**
Conza 5%EC	75	72.59 $^{d}_{(58.43)}$	87.64 $^{b}_{(69.47)}$
	150	100 $^{a}_{(88.35)}$	100 $^{a\,\delta}$ $_{(88.35)\,\beta}$
	300	100 $^{a}_{(88.35)}$	100 $^{a}_{(88.35)}$
Cabrio*^{Top}	75	0.00$^{h}_{(1.65)}$	0.00$^{h}_{(1.65)}$
	150	0.00$^{h}_{(1.65)}$	4.80 $^{g}_{(12.66)}$
	300	0.00$^{h}_{(1.65)}$	7.62 $^{f}_{(15.99)}$
Provax 200WP	75	64.81 $^{e}_{(53.62)}$	82.63 $^{c}_{(65.39)}$
	150	100 $^{a}_{(88.35)}$	100 $^{a}_{(88.35)}$
	300	100 $^{a}_{(88.35)}$	100 $^{a}_{(88.35)}$
Bavistin 50WP	75	83.33 $^{c}_{(65.94)}$	100 $^{a}_{(88.35)}$
	150	86.66 $^{b}_{(68.69)}$	100 $^{a}_{(88.35)}$
	300	100 $^{a}_{(88.35)}$	100 $^{a}_{(88.35)}$
Dithane M-45	75	28.88 $^{g}_{(32.50)}$	35.85 $^{e}_{(36.78)}$
	150	42.59 $^{f}_{(40.75)}$	55.28 $^{d}_{(48.04)}$
	300	65.55 $^{e}_{(54.06)}$	80.96 $^{c}_{(64.14)}$
Control		90 mm	0.438 g

$^{\beta}$ Figures within the parentheses are the transformed (arcsine) values. $^{\delta}$ Means within the same column followed by a common letter(s) are not significantly different (α = 0.05).

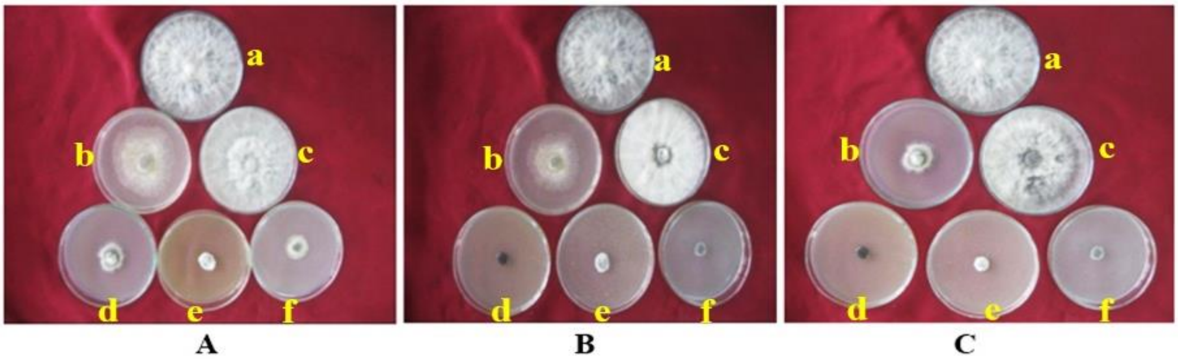

Fig. 4. Effect of fungicides at different concentrations on radial growth of *M. phaseolina* isolate MSP-4 (A-C, Where A= 75 ppm; B= 150 ppm; C= 300 ppm; a= Control; b= Dithane M-45; c= Cabrio^{*Top}; d= Provax 200WP; e= Bavistin 50WP; f= Conza 5%EC).

In-vitro evaluation of organic amendments against *M. phaseolina* isolate MSP-4:
Another experiment was conducted to evaluate the effect of different organic amendments on the growth and development of *M. phaseolina* isolate MSP-4. The highest reduction (50.92%) in radial colony diameter was achieved with mustard oil cake at 3% concentration followed by 2% soybean oil cake (25.18%), and 2% mustard oil cake. All other amendments were found least effective against *M. phaseolina*. Around null inhibition of radial growth was observed with all concentrations of tea waste and 1% conc. of coconut, and sesame oil cake. Besides, evaluation of mycelial dry weight the highest (55.82%) inhibition was observed with 3% mustard oil cake followed by 3% conc. of sesame oil cake, whereas tea waste was found the lowest effect on mycelial dry weight (**Table 6 and Fig. 5**). This result of the present investigation revealed that mustard oilcake was the most effective in inhibiting the growth of *M. phaseolina*.

Table 6. *In-vitro* evaluation of organic amendments against test pathogen

Organic amendments	Concentration (%)	% Inhibition in *M. phaseolina* isolate MSP-4	
		Radial growth	Mycelial dry weight
Mustard oil cake	1	$11.85^{de}_{(20.13)}$	$24.53^{d}_{(29.69)}$
	2	$19.62^{bc}_{(26.26)}$	28.68 ^{c} ð $_{(32.36)}$ ß
	3	$50.92^{a}_{(45.52)}$	$55.82^{a}_{(48.34)}$
Sesame oil cake	1	$0.00^{f}_{(1.65)}$	$5.35^{j}_{(13.38)}$
	2	$11.48^{de}_{(19.80)}$	$21.65^{e}_{(27.73)}$
	3	$17.22^{c}_{(24.49)}$	$35.30^{b}_{(36.45)}$
Soybean oil cake	1	$9.62^{e}_{(18.06)}$	$12.10^{h}_{(20.34)}$
	2	$17.59^{c}_{(24.79)}$	$18.94^{f}_{(25.80)}$
	3	$25.18^{b}_{(30.11)}$	$30.48^{c}_{(33.51)}$
Coconut oil cake	1	$0.00^{f}_{(1.65)}$	$3.85^{k}_{(11.31)}$
	2	$8.51^{e}_{(16.94)}$	$13.73^{g}_{(21.74)}$
	3	$15.18^{cd}_{(22.91)}$	$18.87^{f}_{(25.75)}$
Tea waste	1	$0.00^{f}_{(1.65)}$	$0.00^{l}_{(1.65)}$
	2	$0.00^{f}_{(1.65)}$	$0.00^{l}_{(1.65)}$
	3	$0.00^{f}_{(1.65)}$	$7.31^{i}_{(15.68)}$
Control		90.00 mm	0.442 g

ß Figures within the parentheses are the transformed (arcsine) values. ð Means within same column followed by a common letter(s) are not significantly different (α = 0.05).

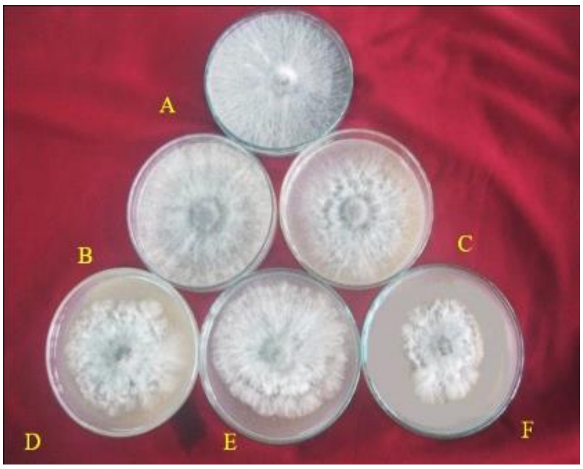

Fig. 5. Effect of organic amendments on radial growth of *M. phaseolina* at 3% concentration (A= Control, B= Tea waste, C= Coconut oil cake, D= Soybean oil cake, E= Sesame oil cake, F= Mustard oil cake).

Integrated effect of bio-agent, fungicide, and organic amendment:

Effect on soybean charcoal rot:

The lowest pre-and post-emergence, as well as total seedling mortality, were found in the treatment T_1 (4.38, 0.00, and 4.38%) where no pathogen was inoculated. But, the highest 80.47% reduction of seedling mortality was recorded in the treatments T_9. On the contrary, significantly the lowest reduction of seedling mortality was observed in the treatment T_5 but identical with T_3 (**Table 7**). Moreover, disease incidence and severity of charcoal rot of soybean were also influenced by the application of bio-agent*,* fungicide and organic amendment either alone or in combination. The lowest disease incidence 6.50% and severity 2.08% was observed in the treatment T_9 followed by the treatments T_6, and T_7 (**Table 8 and Fig. 6**).

Table 7. Effect of bio-agent*,* fungicide, and organic amendment on seedling mortality of soybean

Treatments$^{\emptyset}$	% Seedling mortality			% Reduction
	Pre-emergence	Post-emergence	Total	
T_1	$4.38_{(12.08)}$	$0.00_{(1.65)}$	$4.38^{f}_{(12.08)}$	-
T_2	$23.68_{(29.12)}$	$14.47_{(22.36)}$	$38.15^{a\,ð}_{(38.14)\,ß}$	-
T_3	$17.54_{(24.76)}$	$11.84_{(20.12)}$	$29.38^{bc}_{(32.82)}$	22.98
T_4	$15.35_{(23.06)}$	$10.52_{(18.93)}$	$25.87^{cd}_{(30.57)}$	32.18
T_5	$20.17_{(26.96)}$	$14.03_{(22.00)}$	$34.21^{ab}_{(35.79)}$	10.32
T_6	$10.96_{(19.33)}$	$3.94_{(11.45)}$	$14.91^{e}_{(22.71)}$	60.91
T_7	$14.03_{(22.00)}$	$5.70_{(13.81)}$	$19.73^{de}_{(26.37)}$	48.28
T_8	$15.78_{(23.41)}$	$7.01_{(15.36)}$	$22.80^{cd}_{(28.52)}$	40.23
T_9	$5.26_{(13.26)}$	$2.19_{(8.51)}$	$7.45^{f}_{(15.84)}$	80.47

Table 8. Effect of bio-agent, fungicide, and organic amendment on charcoal rot disease of soybean

Treatments$^{\emptyset}$	% disease incidence	PDI	% Disease reduction	
			Incidence	PDI
T_1	$0.00^{i}_{(1.28)}$	$0.00^{c\,ð}_{(1.28)\,ß}$	-	-
T_2	$32.73^{a}_{(34.89)}$	$18.17^{a}_{(25.22)}$	-	-
T_3	$25.20^{c}_{(30.13)}$	$11.88^{b}_{(20.14)}$	23.00	34.61
T_4	$22.00^{d}_{(27.97)}$	$11.52^{b}_{(19.81)}$	32.78	36.59
T_5	$29.35^{b}_{(32.80)}$	$13.96^{ab}_{(21.90)}$	10.32	23.17
T_6	$12.79^{g}_{(20.95)}$	$9.07^{b}_{(17.50)}$	60.92	50.08
T_7	$16.83^{f}_{(24.22)}$	$9.76^{b}_{(18.18)}$	48.57	46.28
T_8	$19.56^{e}_{(26.24)}$	$11.17^{b}_{(19.50)}$	40.23	38.52
T_9	$6.50^{h}_{(14.77)}$	$2.08^{c}_{(5.68)}$	80.14	88.55

^{ß} Figures within the parentheses are the transformed (arcsine) values. ^{ð} Means within same column followed by a common letter(s) are not significantly different ($\alpha = 0.05$).

A B

Fig. 6. Symptoms of charcoal rot disease at soybean field (A-B, Where A= Seedling stage B= Mature stage).

Effect on yield and yield components of soybean:

The highest plant height, number of pods per plant and seed weight were observed in the treatment T_9 followed by T_1, and T_6. Growth promotion in the treatment T_3 appeared significantly the most inferior in comparison to the other treatments and identical to the control-2 treatment. In the case of disease reduction, the treatment T_5 was significantly inferior to the treatment T_3 but significantly higher in improving the growth promotion parameter. Different treatments also showed significant variation in total yield over control-2. The highest (2.31 t/ha) seed yield was obtained from the treatment T_9, where colonized *T. harzianum*, Provax 200WP treated seed and mustard oil cake were used. Considerably higher and statistically similar seed yield over control-2 was obtained in the treatmentT_1, T_6, T_7, and T_8, respectively. On the other hand, the lowest yield (1.13 t/ha) was obtained in the treatment T_2 where fresh seeds were sown in *M. phaseolina* isolate MSP-4 inoculated soil without application of any means of control (**Table 9**).

Table 9. Effect of bio-agent, fungicide, and organic amendment on soybean yield production

Treatments ø	Plant height (cm)	No. of pod/ plant	Yield (t/ha)	% Increased
T_1	74.77 cd	50.44 ef	2.06 ab δ	-
T_2	69.37 e	45.78 g	1.13 f	-
T_3	73.53 d	49.22 f	1.47 e	30.09
T_4	74.60 cd	51.78 de	1.70 cde	50.44
T_5	77.27 bc	54.00 bc	1.54 de	36.28
T_6	76.57 bc	52.56 cd	1.86 bc	64.60
T_7	78.57 ab	55.11 b	1.81 bcd	60.18
T_8	78.20 ab	54.22 bc	1.74 cde	53.98
T_9	80.67 a	58.67 a	2.31 a	104.42

δ Means within same column followed by a common letter(s) are not significantly different (α = 0.05).

øT_1= Fresh seeds sown in sterilized (5% formaldehyde) soil (Control-1), T_2= Soil inoculated with pathogen (SIP) + Fresh seeds (Control-2), T_3= SIP + Fungicide Treated Seeds (FTS), T_4= SIP + Wheat Grains Colonized *T. harzianum* isolate ISR-26 (WGCT) + Fresh seeds, T_5= SIP+ Organic Amendment (OA) + Fresh seeds, T_6= SIP+ WGCT + FTS, T_7= SIP+ WGCT + OA + Fresh seeds, T_8= SIP+ OA + FTS, and T_9= SIP+ WGCT + OA + FTS.

DISCUSSION

In Bangladesh, this is the first comprehensive study that aimed to develop a sustainable integrated management package against the charcoal rot disease of soybean and surge the yield production at open field condition. Before going to the field trial, a series of preliminary experiments were conducted to select a virulent isolate of *M. phaseolina*, an effective antagonistic isolate of *Trichoderma* sp*.,* suitable fungicide and organic amendment. Exactly seven isolates of *M. phaseolina* were isolated from naturally infected crops and characterized based the morphological features such as pycnidia, pyncidiospores, conidiophores, etc. (Wheeler, 1975). In addition, the pathogenicity test was performed in pot culture with the isolates of *M. phaseolina*. Among the 7 isolate of *M. phaseolina*, isolate MPS-4 was found highly virulent and caused 74.06% total soybean seedling mortality. The results of the pathogenicity test are completely agreed with Hashem (2004) who observed the ability of *M. phaseolina* to infect soybean roots and produced the symptoms of damping-off and root-rot diseases. On the contrary, 87 isolates of *T. harzianum* were screened against *M. phaseolina* isolate MPS-4. Among the 87 isolates, *T. harzianum* isolate ISR-26 showed the highest inhibition of radial growth (81.85%) of test pathogen. The study of the screening of *T. harzianum* isolates against the major soil borne pathogens such as *Fusarium oxysporum* f. sp*. lentis*, *Rhizoctonia solani* and *Sclerotium rolfsii* by dual culture technique were reported by other investigators (Das *et al.,* 2019; Rubayet *et al.,* 2020). Another trial was implemented on five fungicides namely, Cabrio*Top, Conza 5%EC, Bavistin 50WP, Dithane M-45, and Provax 200WP at three different concentrations viz., 75, 150, and 300 ppm *in-vitro* against *M. phaseolina* isolate MPS-4 for selecting the most effective fungicide with appropriate concentration. The results of the present study revealed that Conza 5%EC was the most effective fungicide against *M. phaseolina* isolate MSP-4 which was followed by Provax 200WP, and Bavistin 50WP, respectively. Further, Provax 200WP was selected for minimizing the inoculum density of test pathogen at field level. Actually, Provax 200WP is a popular seed treating fungicide which has a great sustainable potentiality to control the soil as well as seed borne pathogens. Seed treating fungicide could play a preventive measure strikingly rather than foliar spray. However, the present experiment is supported by Pant and Mukhopadhayay (2001). They observed that Provax 200WP (Vitavax) had highly inhibitory against the *R. solani, S. rolfsii* and *M. phaseolina*, and no inhibitory effect on biocontrol agents such as *T. harzianum.* The last preliminary laboratory evaluation was the determination of effective organic amendment against the test pathogen. Among the five-organic amendment, mustard oil cake was found to be most effective and significantly superior to all other organic amendments in inhibiting the growth of *M. phaseolina*. Sesame oil cake and soybean oil cake also had a considerable effect in reducing the growth of selected pathogen but tea waste and coconut oilcake was found less effective against pathogen. This result of the present investigation revealed that mustard oilcake was the most effective in inhibiting the growth of *M. phaseolina* and had a significant impact on yield production of soybean. Sudha and Prabhu (2008) found that, farmyard manure @ 12.5 t/ha and neem cake @ 250 kg/ha was declined the inoculum levels of *M. phaseolina* and significantly reduced the incidence of charcoal rot of sunflower to 13.30 and 15.00%, respectively from 63.30% in the control.

Finally, an integrated management trial was conducted against the *M. phaseolina* isolate MPS-4 inoculated soil condition. The integrated management trial was composed of wheat grain colonized *T. harzianum* isolate ISR-26, mustard oil cake and seed treatment with Provax 200WP fungicide. Integration of *Trichoderma* with Provax 200WP and mustard oil cake under treatment T_9 was appeared to be the best and significantly superior in comparison to other treatments in reducing the seedling mortality, disease incidence and disease severity and increasing the yield of soybean. Rubayet and Bhuiyan (2016) reported that, fungicide, organic amendment and *T. harzianum* either individual or in combination of wheat grain colonized *T. harzianum* @ 90 g/m^2 with Provax 200WP @ 0.02% treated seed and mustard oilcake @ 60 g/m^2 was significantly decreased the pre- and post-emergence mortality of potato seedling caused by *S. rolfsii* and also significantly increased the yield of potato tuber. On the other hand, bio-agent (*T. harzianum,* isolate T-2), fungicide (Iprodione, Rovral 50WP) and plant leaf extract (*Azadirachta indica*) was reduced strikingly the *Alternaria* blight diseases of radish caused by *Alternaria brassicae* AB-2 isolate (Arefin *et al.,* 2019). Results of the present study indicated that the application of different treatments in the field; seed yield and yield contributing components were significantly increased by all the treatments over treatment T_2. Statistically, the highest yield was found with the treatment T_9. The yield increased not only because of declining plant disease but also might be due to the secretion of different growth-promoting substances in the soil by bio-agent *T. harzianum*. According to Altomare *et al.* (1999) reported that *Trichoderma* produced various chemical substances which are accelerated to solubilize minerals, for instance, rock phosphate, Zn, Mn^{4+}, Fe^{3+}, Cu^{2+} etc. and increased iron availability. These nutrient substances might be subsidized in increasing crop yield. Besides, seed treatment was found more effective than foliar spray in case of individual or integrated application of chitosan and *T. harzianum* spore suspension for the protection of southern blight disease and also increases the yield of carrot (Ahmed *et al.,* 2019). Moreover, *T. harzianum* based treatment at different formulation was appeared to be an excellent bio-agent in controlling anthracnose disease of chilli caused by *C. capsici* as well as significantly increased of growth and yield (Simi *et al.,* 2019).

CONCLUSION

The result of the present study revealed that integrated use of bio-agent (*T. harzianum* isolate ISR-26), seed treating fungicide (Provax 200WP), and organic amendment (Mustard oil cake) delivered the effective control measure against charcoal rot disease of soybean caused by *M. phaseolina* isolate MSP-4. Moreover, this technique may be a sustainable alternative to reduce the pathogen population density as well as increasing soybean production at field soil.

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المكافحة المتكاملة لمرض العفن الفحمى لفول الصويا الناجم عن ماكروفومينا فاصولينا

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الملخص العربى

تم إجراء محاولة لإدارة مرض العفن الفحمى لفول الصويا الناجم عن ماكروفومينا فاصولينا باستخدام تكامل العامل الحيوي مع مبيد الفطريات والتعديل العضوي. قبل الذهاب إلى التجارب الميدانية ، أجريت تجارب مختلفة في المختبر لاختيار عزلة ممرضة من *M. phaseolina* ، وكذلك عزلة مضادة لها من *Trichoderma harzianum* ، ومبيد فطري مناسب ومحسنات عضوية للتربة. تم اختيار عزلة MSP-4من بين سبع عزلات من *M. phaseolina* كنتيجة لاختبار القدرةالإمراضية. على العكس من ذلك ، من بين سبعة وثمانين عزلة من *T. harzianum* ، أظهرت عزلة ISR-26 أعلى تثبيط (81.85٪) للنمو الفطرى للفطر ماكروفومينا. في حالة تجربة تقييم مبيدات الفطريات ، تم العثور على Provax 200WPأكثر مبيدات الفطريات فعالية في معالجة البذور بتركيز بمعدل (150 جزء في المليون) ، بينما شهد أعلى تركيز لـ Conza 5EC و Bavistin 50WP لتثبيط النمو الفطرى لعزلة M. phaseolina MSP-4.بالإضافة إلى ذلك ، تم إجراء تجربة في المختبر لتعديلات عضوية مختلفة ، وجدت أن كعكة زيت الخردل هي التعديل العضوي الأكثر نشاطًا لتقليل النمو (50.92٪) وتطوير مسببات الأمراض عند مستوى تركيز 3٪. في التجربة الحقلية ، ظهر الاستخدام المتكامل لـ *T. harzianum* مع Provax 200WPوكعكة زيت الخردل تحت المعاملة تريكودرما T9 أفضل علاج في تقليل موت الشتلات (80.47٪) ونسبة الإصابة بالمرض (80.14٪) وكذلك شدة المرض (88.55٪) التي يسببها الفطر الممرض. علاوة على ذلك ، كانت المعاملة تريكودرما T9 هي أفضل علاج ليس فقط للتحكم في مرض العفن الفحمى لفول الصويا ولكن أيضًا زادت الكمية المعنوية من المحصول (104.42٪). و قد يكون هذا النهج المتكامل أكثر فائدة لمزارعي فول الصويا في جميع أنحاء العالم.

الكلمات المفتاحية: ماكروفومينا فاصولينا، تريكودرما هارزينم، مبيدات الفطريات، محسنات عضوية، وفول الصويا