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Utilizing the combined antifungal potential of *Trichoderma* spp. and organic amendments against dry root rot of mungbean

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Abstract

The present study was carried out to evaluate the effect of bioagents and organic amendments in suppressing the dry root rot of mungbean incited by *Rhizoctonia bataticola*. The locally isolated pathogen and fungal biocontrol agents were identified based on morphological and molecular characterization. These identified bioagents were tested in vitro, and the highest mycelial inhibition was recorded in dual culture assay by *Trichoderma harzianum* (74.44%), and among organic amendments, maximum mycelial inhibition was found in neem cake (61.11%). In a greenhouse study, *T. harzianum* + neem cake effectively enhanced the percent germination (93.33%) and decreased the percent disease mortality (11.67%) than the other treatments. The morphological parameter like plant height (57.50 cm), dry weight (22.83 g) root nodules (51), pods/plant (58), and 100-seed weight (5.78 g) were found to be at the maximum in this combined application. Physiological pigments viz. chlorophyll (2.41 mg/g) and carotenoids (0.19 mg/g), protein content (5.85 mg/g), and leghemoglobin (11.75 mg/g) were also found to be maximum in *T. harzianum* + neem cake and minimum phenol content (1.41 mg/g). The study concludes that *T. harzianum* + neem cake can be recommended as an effective approach for the management of dry root rot of mungbean.

Keywords: Mungbean, Dry root rot, *Rhizoctonia bataticola*, Bioagents, Organic amendments

Background

Mungbean, *Vigna radiata* (L.) Wilczek, is one of the most essential and widely cultivated pulse crops. The crop is attacked by numerous diseases caused by fungi, bacteria, and viruses. Among these diseases is the dry root rot of mungbean that caused by *Rhizoctonia bataticola* (Taub) Butler (Pycnidial stage: *Macrophomina phaseolina* (Tassi.) Goid) and is one of the most devastating diseases occurring in tropical and subtropical countries. This disease causes substantial losses of mungbean causing seed infection ranging from 2.2–15.7% which leads to decrease in the grain yield by 10.8% as well as protein content (12.3%) in seeds (Kaushik et al., 1987).

Phytopathogenic fungi are usually managed using synthetic fungicides, but their applications are limited due to an adverse effect on environment and health. Due to their harmful effects and development of resistance in crop pathogens, the biological-based alternatives were used to manage the diseases of various crops. Bioagents served as an ecologically safe and acceptable substitute for the fungicidal management of soil-borne diseases in recent years (Abada and Ahmad, 2014). Therefore, *Trichoderma* spp. have been used as a microbial antagonist for the management of root diseases of various field crops (Padamini, 2014). Another approach to suppress the soil-borne diseases is to use organic amendments (Bonanomi et al., 2018). Pandey et al. (2011) reported that when fungal antagonists were used in combination with organic amendments, their antagonistic efficacy was enhanced. These bio-intensive methods can be used to keep the economic threshold level below without harming the agroecosystem of soil and also promoting the growth and productivity of mungbean.

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The main aim of the present study was to evaluate the overall efficacy of organic amendments + bio-agent to control the dry root rot disease in a greenhouse experiment.

Materials and methods

Isolation of the casual pathogen and bioagents

Infected roots and rhizospheric soil around the healthy roots were collected from the fields of mungbean. The phytopathogen, *R.bataticola* (Barnett & Hunter, 1972), was isolated from excised diseased root pieces on potato dextrose agar medium (PDA). The pure culture of pathogen was made by hyphal tip isolation method, and typical black mycelial growth of *R. bataticola* was observed after 72 h of incubation, at 25 ± 1 °C. Biocontrol agents were isolated from the rhizosphere of healthy plants by serial dilutions method. *Trichoderma* spp. were compared with identification key given by Rifai (1969) and the pathogen given by Dhingra and Sinclair (1978).

Identification of pathogen and bioagents

The identification of *R. bataticola* and *Trichoderma* spp. was further confirmed by sequencing internal transcribed spacer (ITS) region of 16S rRNA of 5.8S ribosomal RNA. The mycelia were harvested and filtered, and the DNA was extracted by the method given by Doyle and Doyle (1987). ITS region amplification was done by using universal primers ITS 1 and ITS 4 by the method of Ausubel et al. (1995) and sequenced using Sanger dideoxy sequencing technology at the MACROGEN Company (Seoul, Republic of Korea). Sequence obtained was analyzed by using the nucleotide BLAST, and the result was deposited at NCBI (Altschul et al., 1990). The accession numbers were obtained from GenBank.

In vitro inhibition of test pathogen by biocontrol agents and organic amendments

Dual culture plate assay

Mycelial disc of pathogen was placed at one end opposite to the *Trichoderma* spp. The plates were then incubated at 28 ± 2 °C for 7 days. The bioassay of antagonists was evaluated on PDA in Petri plates by dual culture method suggested by Asran-Amal et al. (2010) using the following formula:

$$L(\%) = \frac{C-T}{C} \times 100$$

where L = growth inhibition of pathogen, C = radial growth of the pathogen in the control plate, and T = radial growth of the pathogen in a treated plate.

Organic extracts

Organic amendments used were mustard cake, neem cake, vermicompost, and farmyard manure. Ten grams of finely powdered amendments was mixed in 300 ml sterilized in 500 ml flasks, and the suspension was boiled for 10 min, shaken for 24 h at 100 rpm at 41 °C. It was briefly centrifuged and filtered through Whatman No. 1 filter paper. One milligram of organic extracts was incorporated into PDA. Plates containing only sterile water (1 ml) were used as the control. Hyphal plugs (5 mm) of *R. bataticola* were placed in the center of Petri dishes.

In vivo study

Mass culture of pathogen and biocontrol agents

The inoculum was prepared by growing pathogen on crushed corn seeds. Corn seeds were filled in 500 ml conical flasks, and these were autoclaved at 20 psi for 2 h. The corn seeds were inoculated aseptically by 4 agar plugs (2-mm diameter each). The flasks were incubated at room temperature (28 + 2 °C) for 15 days and shaken occasionally for uniform colonization. Inoculum of each fungal antagonist was also prepared on crushed corn seeds in the same way described before. The inoculum thus obtained was used for the experiments.

Greenhouse experiment

To evaluate the suppression of the root rot of mungbean, 2 biocontrol agents and 2 organic amendments were selected on the basis of the in vitro studies. The experiment was carried out in earthen pots (9 × 12 inches) filled with sterilized sandy soil mixture. The pots were artificially inoculated by *R. bataticola* (100 g/kg soil) and mixed thoroughly up to 5–7-cm depth in the pot and incubated for 5 days for colonization of fungus in the soil. In each pot, 6 surface sterilized seeds (SML-668) were sown. Apparently healthy surface sterilized seeds of mungbean were coated with *T. harzianum* and *T. viride* @ 4 g/kg seeds separately. The organic manure (mustard cake and neem cake) were thoroughly mixed in each pot at 10 percent (w/w) of soil before 1 month of sowing. Surface-sterilized soil inoculated with pathogen inoculums and without any treatment was treated as a control. The treatment in pots was replicated thrice, and the observations were recorded on 40 and 60 DAS.

Growth, yield, and physiological parameters

The seed germination and percent disease mortality were determined after 10 and 25 days, respectively. Growth and yield parameters including the height of plants (cm), dry weight of plants (g/plant), number of root nodules, pods per plant, and 100-seed weight were recorded after 80 DAS. The physiological parameters were quantified using a spectrophotometer (UV 2450, Shimadzu Japan). The leaf content of photosynthetic

pigments (total chlorophyll and carotenoids) was determined using fresh leaf samples. Leaf sample (0.5 g) was homogenized by acetone (90% v/v), filtered, and made to a final volume of 50 ml. Pigment concentrations were calculated from the absorbance of the extract (645 and 663 nm) using the method of Musheer et al. (2019).

To calculate the total chlorophyll, content Arnon's equation (1949) was used.

$$\text{Total chl (mg/g fresh weight)} = \frac{[20.2\ (A_{645}) + 8.02\ (A_{663})]}{1000 \times W} \times V$$

where A = absorbance of light at a particular wavelength, W = weight of the leaf tissue used, and V = final volume of the extract.

To calculate the total carotenoid, the content formula given by Hendry and Price (1993) was used.

$$\text{Total carotenoid (mg/g leaf tissue)} = \frac{[A_{480} + (0.114 \times A_{663}) - (0.638 - A_{645})]}{1000 \times W} \times V$$

Total protein in leaves was determined according to Bradford's method (Bradford, 1976). The total phenolic content (mg/g dry weight of leaves) was calculated from the standard curve and expressed as milligram catechol equivalent of phenol per gram sample at 650 nm according to the method of Khaledi and Taheri (2016). The leghemoglobin content of fresh, bold, and pink nodules was determined by the method of Wilson and Reisenauer (1963) with Drabkin's solution using the following formula:

$$\text{Lb conc.(mg/g)} = \frac{A_{556} - A_{539} \times 2D}{23.4}$$

where D is the initial dilution.

Soil microflora propagules

The initial and final count of population dynamics of *R. bataticola* was estimated at 7 DAS and 45 DAS according to Veena and Reddy (2016) using dilution plating on agar. From each pot, 1 g soil sample was taken in 9 ml sterilized distilled water, and serial dilution was made up to 10^{-4}. An aliquot of 0.5 ml was spread in a Petri plate and incubated in dark at 22–26 °C. The experiment was replicated thrice, and readings were taken after 5 days by visual quantification of colony-forming units per plate.

Statistical analysis

All the experiments were laid in a randomized block design (RBD) in triplicates. The analysis was carried out by analysis of variance (ANOVA) using R software. The least significant difference (LSD) at $P < 0.05$ was used to compare the means. The treatment means were compared by Tukey HSD test.

Result and discussion

Isolation and identification of pathogen and *Trichoderma* spp.

On the basis of preliminary microscopic examination based on morphological characteristics, *Trichoderma* spp. were identified as *T. harzianum*, *T. asperellum*, *T. virens*, and *T. viride*. Similarly, the isolated pathogen was identified as *R. bataticola*. The size of the amplicons was found 550–582 bp for *Trichoderma* spp. and 372–540 bp for *R. bataticola* species. The sequences were blasted against the BLASTN program of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The resulting sequences of nucleotide showed up to 99% homology compared with the database of subunit rDNA and ITS sequence of *R. bataticola* and *Trichoderma* spp. After that, the isolates were sequenced and accession numbers were obtained from GenBank with MK765032 (*R. bataticola*), MK765028 (*T.harzianum*), MK764992 (*T. viride*), MK774725 (*T. virens*), and MK765012 (*T. asperellum*).

In vitro inhibition of test pathogen by bioagents and organic amendments:

T. harzianum (23 mm) was found to be the highly effective in inhibiting the fungal growth of the pathogen, followed by *T. viride* (24.33 mm), *T. virens* (28 mm), and *T. asperellum* (30 mm) as compared to the control (90 mm) (Fig. 1). These findings are also in support of Kumari et al. (2012) who found *T. harzianum* to be most effective in reducing the mycelial growth of pathogen followed by *T. viride*. In dual culture test, mycelial growth of test fungi was inhibited by *Trichoderma* spp. due to release of various diffusible volatiles and non-volatile compounds in the medium like harzianic acid, heptelidic acid, tricholin, glisoprenins, and viridin (Rini and Sulochana, 2007) or their greater ability to compete for space and nutrients (Devi et al., 2012). The results of the inhibitory effect of organic extracts on the fungal growth are indicated in Fig. 2. Neem cake (35 mm) was found to be most effective in inhibiting the mycelial growth of the pathogen followed by mustard cake (44 mm), vermicompost (53.33 mm), and farmyard manure (65.67 mm). Similar finding was observed by Meena et al. (2014) who found the neem cake exhibited a maximum inhibition of mycelial growth of *M. phaseolina* infecting jute. Neem cake possesses fungicidal property due to chemical constituent such as azadirachtin, nimocin, nimolicinol, azadirachtol, and isolimocinolide having fungicidal properties acting on pathogen, thus minimizing the disease (Dubey and Kumar, 2003).

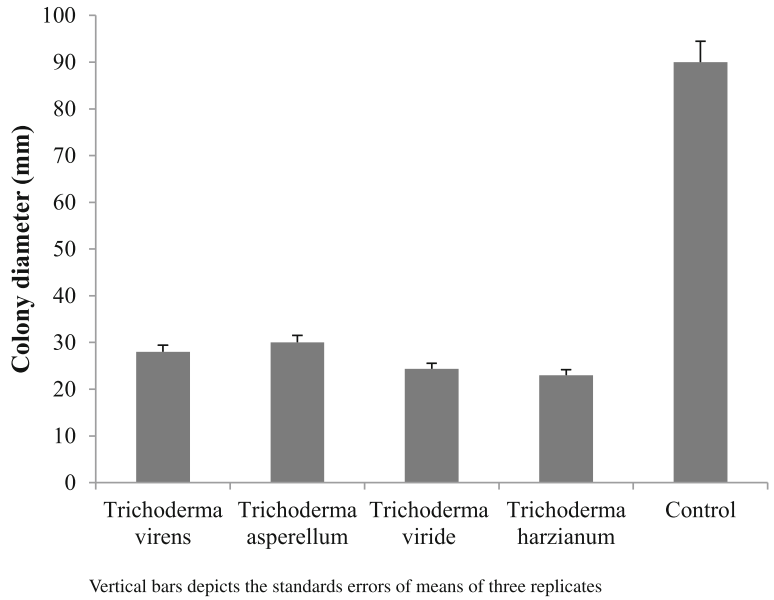

Fig. 1 Effect of the tested bioagents on the mycelial growth of *Rhizoctonia* bataticola

In vivo

Considering the larger inhibition zone provided by the bioagents like *T. harzianum*, *T. viride*, and among organic amendments, neem cake and mustard cake, their subsequent use in managing this disease appeared to be adequate and appropriate for the greenhouse study.

Percentages of germination and disease intensity

Obtained results of pot experiment indicated that all the bioagents and organic amendments increased the percent germination and inhibited the percent disease mortality as shown in Table 1. *T. harzianum* with neem cake was found to be the most effective one than other treatments as well as exhibiting the maximum germination percentage (93.33%) and minimum disease mortality (11.67%) as compared to control. Among sole treatments, *T. harzianum* significantly increased the germination percentage (71.67%) and reduced the disease mortality (40%) followed by *T. viride*. The results were in agreement with Khalili et al. (2016) who reported that mycelial growth of *M. phaseolina* was effectively inhibited by *Trichoderma* spp. and decreased the disease incidence in the pot experiment. Our observations were in agreement with the findings of Lodha et al. (2002) who

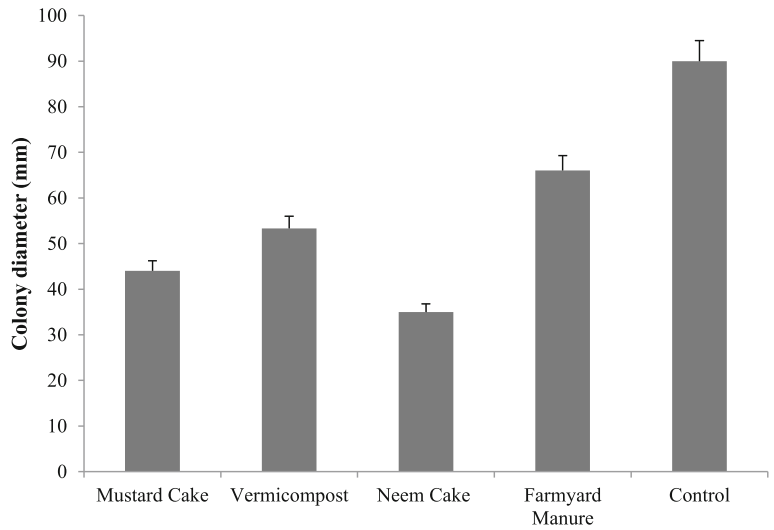

Fig. 2 Effect of the tested organic amendments on the mycelial growth of *Rhizoctonia*

Table 1 Effect of the application of the tested bioagents and organic amendments on germination percentage and percent disease mortality

Treatments	% Germinated seeds	% Disease mortality	% Disease reduction
Trichoderma harzianum	76.67^{d}	38.33^{c,d}	56.36
Trichoderma viride	71.67^{c,d}	40.00^{c}	58.18
Neem cake	63.33^{e}	41.67^{c}	54.54
Mustard cake	58.33^{e,f}	60.00^{b}	34.54
Trichoderma harzianum + neem cake	93.33^{a}	11.67^{e,f}	87.26
Trichoderma harzianum + mustard cake	91.67^{a}	18.33^{f}	80.00
Trichoderma viride + neem cake	88.33^{a,b}	26.67^{e}	70.90
Trichoderma viride + mustard cake	81.67^{b,c}	28.33^{d,e}	69.09
Control	53.33^{f}	91.67^{a}	–
SEM	2.36	3.14	
LSD (P ≤ 0.05)	4.08	5.44	

Values of means with different letters within the columns showed the significant difference at ($P \leq 0.05$) determined by Tukey's HSD test.

reported that soil amendments with compost decreased the population density of *M. phaseolina*, thus reducing the dry root rot severity. Similar results of neem cake efficacy were also obtained by Lakharan et al. (2018). Rajani and Parakhia (2009) reported a combined application of neem cake and *T. harzianum* for effectively managing the root rot disease of castor.

Effect of tested bioagents and organic amendments on the propagule density of pathogen

Bioagents and organic amendments led to increase the level of antagonists result in a decrease of the propagule density of pathogen. This is due to the competition with other microbes or fungitoxic compounds released by them. Various treatments recorded influence on the initial and final count of population dynamics and inoculum density per gram of rhizospheric soil (Fig. 3). The population of *R. bataticola* was reduced effectively by all the treatments than the control (8×10^4, 9.33×10^4 cfu/g soil). Among bioagents, *T. harzianum* (6.66×10^4, 4.66×10^4 cfu/g soil) was the superior treatment that showed the minimum number of colonies, and among organic

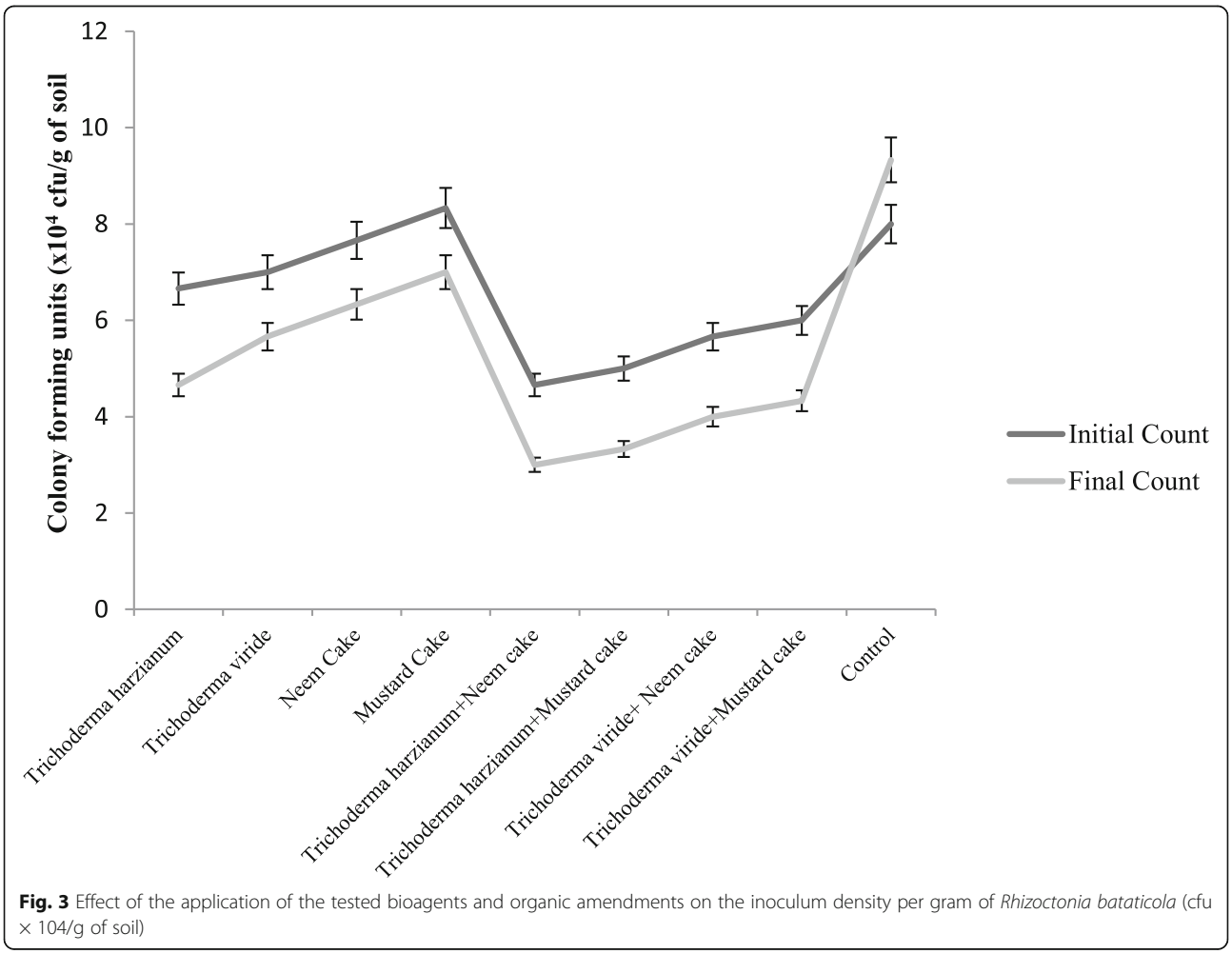

Fig. 3 Effect of the application of the tested bioagents and organic amendments on the inoculum density per gram of *Rhizoctonia bataticola* (cfu × 104/g of soil)

Table 2 Effect of the application of the tested bioagents and organic amendments on the morphological and crop parameters of mungbean

Treatments	Plant height (cm)	Plant dry weight (g)	No. of root nodules	No. of pods/plant	100-seed weight (g)
Trichoderma harzianum	47.50 ± 0.29	16.16 ± 0.17	45.33 ± 0.33	35.00 ± 0.88	5.26 ± 0.02
Trichoderma viride	46.67 ± 0.67	15.50 ± 0.29	42.67 ± 0.33	29.67 ± 1.73	5.15 ± 0.02
Neem cake	43.17 ± 0.17	13.50 ± 0.29	39.00 ± 0.58	22.00 ± 1.53	4.78 ± 0.01
Mustard cake	40.33 ± 0.33	13.33 ± 0.33	37.67 ± 0.67	17.00 ± 0.58	4.51 ± 0.04
Trichoderma harzianum + neem cake	57.50 ± 0.29	22.83 ± 0.17	51.00 ± 0.58	58.00 ± 1.15	5.78 ± 0.08
Trichoderma harzianum + mustard cake	54.33 ± 0.33	20.83 ± 0.44	50.67 ± 0.33	52.67 ± 0.88	5.73 ± 0.01
Trichoderma viride + neem cake	52.83 ± 0.44	17.83 ± 0.44	49.00 ± 0.58	46.67 ± 0.88	5.59 ± 0.03
Trichoderma viride + mustard cake	50.67 ± 0.67	17.50 ± 0.29	46.67 ± 0.33	42.00 ± 0.58	5.40 ± 0.01
Control	37.00 ± 0.58	12.50 ± 0.29	37.33 ± 0.88	12.33 ± 0.88	4.33 ± 0.01
SEM	0.63	0.44	0.76	1.52	0.03
LSD (P ˂ 0.05)	1.09	0.76	1.31	2.64	0.50

amendments, neem cake reduce the propagule (7.66×10^4, 6.33×10^4 cfu/g soil). Antibiotics like trichodermin, trichodermol A, harzianolide, viridin, and gliotoxin produced by *Trichoderma* spp. helped in suppressing the disease through reduction of germination and production of spore (Khan and Anwer, 2011). Organic amendments are effective in suppressing the pathogenic fungi (Singh and Singh, 1982), and neem cake was found to show a positive effect on the fungal pathogen as shown by Elnasikh (2011). The combined application of *T. harzianum* and neem cake reduced the colony count (4.66×10^4, 3×10^4 cfu/g soil) and significantly superior over other treatments.

Effect of the tested bioagents and organic amendments on growth and yield parameters of mungbean

All the bioagents and organic manure increased the morphological and yield parameters significantly over control (Table 2). *T. harzianum* + neem cake was the most superior treatment which increased the plant height (57.50 cm), dry weight (22.83 g), no. of nodules (51), pods/plant (58), and test weight (100-seed weight) (5.78 g) than the control. Among the sole treatment, maximum plant height (47.50 cm), dry weight (16.16 g), no. of nodules (45.33), pods/plant (35), and test weight (100-seed weight) (5.26 g) were recorded in *T. harzianum* followed by *T. viride*. Root colonized with biocontrol agents not only suppresses the disease but also helps in increasing the growth of plant (Gautam et al., 2015) by increasing the uptake of nutrients by roots or by releasing the plant hormones (Harman et al., 2004). In addition, Srivastava et al. (2010) also stated that when *Trichoderma* spp. interact with plants, it results in the promotion of growth, enhances nutrient availability, increases crop yield, and improves disease resistance. The use of organic amendments in reducing disease intensity and increasing grain yield has been reported by several

Table 3 Effect of the application of the tested bioagents and organic amendments on the physiological parameters of mungbean grown in soil infested with *Rhizoctonia bataticola*

Treatment	Physiological pigments (mg/g)		Phenol content (mg/g DW)	Leghemoglobin content (mg/g)	Protein content (mg/g)
	Total chlorophyll content	Carotenoid content			
Trichoderma harzianum	2.09 ± 0.06	0.28 ± 0.03	1.69 ± 0.05	4.93 ± 0.01	10.88 ± 0.04
Trichoderma viride	1.76 ± 0.01	0.24 ± 0.01	1.75 ± 0.01	4.51 ± 0.06	10.46 ± 0.08
Neem cake	1.61 ± 0.02	0.16 ± 0.08	1.85 ± 0.05	4.15 ± 0.03	9.71 ± 0.07
Mustard cake	1.54 ± 0.01	0.16 ± 0.02	1.98 ± 0.03	3.85 ± 0.01	9.26 ± 0.04
Trichoderma harzianum + neem cake	2.41 ± 0.08	0.19 ± 0.02	1.41 ± 0.08	5.85 ± 0.01	11.75 ± 0.02
Trichoderma harzianum + mustard cake	2.36 ± 0.17	0.28 ± 0.01	1.47 ± 0.05	5.75 ± 0.01	11.63 ± 0.01
Trichoderma viride + neem cake	2.21 ± 0.06	0.27 ± 0.01	1.53 ± 0.08	5.42 ± 0.03	11.26 ± 0.01
Trichoderma viride + mustard cake	2.16 ± 0.01	0.26 ± 0.08	1.62 ± 0.01	5.32 ± 0.06	11.09 ± 0.08
Control	1.39 ± 0.04	0.15 ± 0.01	2.04 ± 0.05	3.66 ± 0.05	8.50 ± 0.01
SEM	0.28	0.05	0.11	0.21	0.64
LSD (P ≤ 0.05)	0.52	0.69	0.17	0.35	1.04

workers (Kapoor et al., 2006). Neem seed cake was found to induce the best growth in plants as confirmed by Babariya et al. (2016).

Effect of the tested bioagents and organic amendments on physiological parameters of mungbean

The data of crop physiological parameters indicated that all the treatments significantly affected the host physiology as reported in Table 3. Leaf pigments like total chlorophyll and carotenoid content are reduced significantly in diseased plants due to choking of xylem vessels caused by wilting, drying, and blighting of leaves by the toxins released by fungus affecting the synthesis of chlorophyll. There are improper functioning and significant reduction of root nodules due to infection of root rot fungus, *M. phaseolina* (Muthomi et al., 2007). The maximum total chlorophyll (2.41 mg/g) and carotenoid content (0.19 mg/g), protein content (5.85 mg/g), and leghemoglobin (11.75 mg/g) were recorded in the combined treatment of *T. harzianum*+ neem cake followed by *T. harzianum*, and mustard cake than the control. The enhancement in total leaf chlorophyll and carotenoid content due to treatment with bioagents and organic amendments is due to decrease in the rotting of roots. Phenol content was significantly reduced in the combined treatment (1.41 mg/g) as compared to control (2.04 mg/g). Among sole treatments, *T .harzianum* was the most effective in increasing the total chlorophyll (2.09 mg/g), carotenoid content (0.28 mg/g), protein content (4.93 mg/g), and leghemoglobin (10.88 mg/g). The present observations of increase in leghemoglobin content in roots by application of bioagents and organic amendments are due to the proper functioning of roots. Phenol content in sole treatments was found to be minimum in the treatment of *T. harzianum* followed by *T. viride*. Similar findings were also reported by Doley and Jite (2013), where the total phenol content was enhanced by the infection of *M. phaseolina* in groundnut plants. Benhamou et al. (2000) reported that phenolic compounds increase the mechanical strength of the host cell wall which reduced the pathogen infection. Inoculation of *Trichoderma* spp. helped in increasing the protein content (Rajik et al., 2012). Trichoderma spp. enhanced the micronutrient availability and adsorption to plants such as Mg, Fe, and Zn which are required for synthesis of photosynthetic pigments (Srivastava et al., 2006). Obtained results were also similar to Rahdari et al. (2012) who reported an increase in chlorophyll content by addition of organic amendments.

Conclusion

The present study demonstrated that using combinations of bioagents and organic amendments for controlling the dry root rot pathogen (*R. bataticola*) showed a potential benefit in managing the disease as well as enhancing the morphological, physiological, and yield parameters. Hence, this low input technology is potentially useful for the management of disease and helpful for the resource-deficient growers of India.

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Authors' contributions

The isolation of pathogen collection and analysis of data was mainly done by AC and was the major contributor in writing the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials

The database sequence of biocontrol agents and pathogen isolated and identified during the current study is submitted in the NCBI GenBank. The data collected and analyzed during the current study is available from the corresponding author.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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