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BIO-EFFICACY OF *TRICHODERMA* ISOLATES AND *BACILLUS SUBTILIS* AGAINST ROOT ROT OF MUSKMELON *CUCUMIS MELO* L. CAUSED BY *PHYTOPHTHORA DRECHSLERI* UNDER CONTROLLED AND FIELD CONDITIONS

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Abstract

Root rot of muskmelon caused by *Phytophthora drechsleri* (Tucker) is considered the most important disease limiting muskmelon production in Pakistan. In this study, three molecularly characterized isolates of *Trichoderma* (*T. harzianum* HK, *T. harzianum* HM, *T. asperellum* TH) and phenotypically characterized isolate of *Bacillus subtilis* were evaluated against *P. drechsleri* under laboratory conditions. The different antagonism assays such as, dual culturing, non-volatile, volatile metabolites and field conditions were tested. The results showed that all tested isolates of *Trichoderma* and *Bacillus subtilis* significantly ($p<0.001$) inhibited the mycelial growth of the pathogen. In all dual cultures, *T. asperellum* TH showed maximum mycelial inhibition followed by *T. harzianum* HK, *T. harzianum* HM and *Bacillus subtilis*. Non-volatile metabolites were more effective against pathogens than volatile metabolites. Under field conditions, percent disease incidence was decreased while percent plant survival, dry root and shoot weight significantly ($p<0.001$) were increased by the application of T_o (combination of all tested *Trichoderma* isolates and *B. subtilis*) followed by *T. asperellum* TH during both growing seasons. It is suggested that bio-control agents can be used for the sustainable management of root rot of muskmelon. (*Cucumis melo* L.)

Key words: Biocontrol agents, *P. drechsleri*, Inhibitory effect, Metabolites, Disease incidence, Plant survival.

Introduction

Root rot caused by *Phytophthora drechsleri* is an economically important disease of muskmelon in Pakistan (Majid *et al*., 1994). This soil-borne pathogen survive as oospores for several years (Jee *et al.,* 2001) and produces zoospores that infect plant tissues and are dispersed by the irrigation water (Zhang *et al.,* 2010). Some agronomic practices like mulching, crop rotation, use of synthetic chemicals and soil moisture management reduced the disease severity (Lamour & Hausbeck, 2000). *Phytophthora* species have different bio-chemical pathways, due to which the chemical fungicides gave no appropriate control (Drenth & Sendall, 2001).

The use of bio-control agents has great potential as an alternative to noxious chemicals. *Trichoderma* and *Bacillus* species are being used as bio-control agents against *Phytophthora* species (Chung *et al.,* 2008; Wang *et al.,* 2013). *Trichoderma* species are known as the best biological control of many fungal plant pathogens, especially pathogens against soil-borne (Elad, 2000; Keswani *et al.,* 2014; Srinivasa & Devi, 2014). *Trichoderma* spp. has different interactional mechanisms like mycoparasitism, competition and antibiotics to antagonize the phytopathogens (Pal & Gardener, 2006). Several species produced nonvolatile and volatile metabolites substances specially tricholin, massoilactone, gliovirin, heptelidic acid, alamethicins, peptaibols, harianic acid and glisoprenins which are lethal to plant pathogens (Qualhato *et al.,* 2013). Antagonistic potential of *Trichoderma* isolates against *P. drechsleri* and *P. cryptogea* have been reported as effective under the laboratory conditions (Moayedi & Ghalamfarsa, 2011). Some bacterial isolates were also reported as effective biocontrolling agents against disease incited by *Phytophthora* (Thanh *et al.,* 2009). Different isolates of *Bacillus subtilis* and *Pseudomonas fluorescens* were antagonistic to *P. drechsleri* (Singh & Dubey, 2010).

Furthermore, applications of chemical fungicides on fruit crops have drawbacks, like harmful residual effects, environment pollution and development of resistance in pathogens. Considering this, there is a need for alternative approaches which are environment friendly, non-toxic to human health and have long-term effectiveness against pathogens. The objective of this study was to evaluate the antagonistic potential of different biocontrol agents against root rot of muskmelon under laboratory and field conditions for its sustainable management.

Material and Methods

Collection, Isolation, and identification of Pathogen: Diseased samples were collected from a muskmelon field located at District Jhang (30°35′N; 71°39′E), Punjab, Pakistan. The samples were kept in zipped (sterile) bags and brought to the laboratory of Department of Plant Pathology, College of Agriculture, University of Sargodha for further processing. Isolation was performed from root samples on PARP medium (corn meal agar 17g L^{-1}, 0.4ml Pimaricin L^{-1}, 0.25g Ampicilline L^{-1}, 0.01g Rifampcin L^{-1}, 5mL Pentachloronitrobenzene (PCNB) L^{-1}, 1mL Dimethylsulphooxide (DMSO) L^{-1}) by using the tissue segment method described by Rangaswamy (1958). The pathogen was identified via cultural and morphological characteristics reported in literature (Bush *et al.,* 2006; Jadesha, 2014). The isolated strains were maintained on PDA (potato starch 4g L^{-1}, agar 20g $L^{-1}$and dextrose 20g L^{-1}) and stored at 4°C for further use.

Trichoderma culture: Three different *Trichoderma* isolates (*T. harzianum* HM, *T. harzianum* HK, and *T. asperellum* TH) were obtained from the Department of Plant Pathology, College of Agriculture, University of Sargodha and maintained on PDA at 24 ±1ºC (Ghazanfar *et al.,* 2016).

Bacterial culture: *Isolation, identification and purification of bacteria:* Soil samples were collected in sterilized polythene zipped bags from the Research Area of the University of Sargodha and brought to the laboratory for isolation using the serial dilution technique (Aneja, 2002; Maleki *et al.,* 2011). Identification was made on the basis of morphology, colony color, Gram staining, and shape under 100X microscope. Crystal violet (CV) stain was used as a staining agent and identification was performed by comparing samples to cultural and morphological characters reported by Schaad *et al.,* (2001).

For the pure culture of *Bacillus*, a single colony was streaked on a sterilized Nutrient agar (NA) (5g Peptone L^{-1}, 3g Beef extract $L^{-1}$and 15g Agar L^{-1}) medium plate and incubated at 28±2°C for 36 hours and preserved at 4ºC for future use.

Antagonistic activity test of *Trichoderma* isolates and *B. subtilis* against *P. drechsleri* in Dual culture test: *Trichoderma* isolates were tested against *P. drechsleri* by using dual culture technique in three different ways; seven days old, 5 mm mycelial bit of pathogenic and antagonist were placed opposite to each other in 90 mm diameter Petri plates which contained sterilized Potato dextrose agar (PDA) media (Dual culture I) one mycelial bit of pathogen (5 mm) placed in the center of Petri plate and three mycelial bits of antagonist (5 mm) were placed in triangle form on equal distance from the antagonist (Dual culture II) and one mycelial bit of antagonist (5 mm) placed in the center of Petri plate and three mycelial bits of the pathogen (5 mm) were placed in triangle form at equal distance from pathogenic fungi (Dual culture III). A mycelial bit of pathogen placed in the center of Petri plates containing sterilized PDA served as control. The Petri plates were incubated at 25±1ºC and examined on regular basis. The dual culture of *Bacillus subtilis* followed the same procedure as *Trichoderma* with only replacing *Trichoderma* bits with *Bacillus* streaks.

The percent inhibition rates were calculated on 3, 5 and 8 days after inoculation by following formula:

Formula 1.

$$\text{Inhibition percentage} = \frac{C - T}{C} \times 100$$

where;
C = mycelial growth of pathogen in control
T = mycelial growth of pathogen in dual culture

Metabolites: *Non-volatile metabolites:* To study the effect of non-volatile metabolites of *Trichoderma* spp. on mycelial growth of *P. drechsleri*, cellophane membranes (9 cm) were used (Dennis & Webster, 1971a; Khalili *et al.,* 2012). Cellophane membranes of 9 cm were autoclaved and placed on 9 cm Petri plates containing sterilized PDA. Seven-days-old, 5 mm mycelial bit of each *Trichoderma* species was placed in the center of different plates above the cellophane membrane and incubated at 24±1ºC. After two days, cellophane sheets along with *Trichoderma* bit were replaced by seven-days-old 5 mm mycelial bit of *P. drechsleri*. Evaluation of *Bacillus subtilis* for non-volatile metabolites was the same as for *Trichoderma*, with *Trichoderma* replaced by a *Bacillus* streak. Petri plates inoculated with a mycelial bit of pathogen were the control. The data for inhibition percentage of the pathogen were recorded after 3, 5 and 8 days by using formula 1.

Volatile metabolite: The effect of volatile metabolites of *Trichoderma* spp. on mycelial growth of *P. drechsleri,* was determined using method of Dennis & Webster (1971b) and Khalili *et al.,* (2012). Seven-days-old 5 mm mycelial bits of *Trichoderma* spp. were placed in the center of different sterilized PDA plates. The plates containing PDA medium were inverted and kept over the plates having *Trichoderma* spp.in sterilized condition, sealed with the Para film and incubated in dark conditions for the period of two days. After two days, the inverted Petri plates were removed and seven-days-old 5 mm mycelial bit of *P. drechsleri* was placed on a PDA plate and incubated at 25±1ºC. Evaluation of *Bacillus subtilis* for volatile metabolites was same as adapted for *Trichoderma* with only replacing the *Bacillus* streak instead of *Trichoderma* bit. Petri plates inoculated with a mycelial bit of *P. drechsleri* were treated as control. Data for inhibition percentage of pathogen were recorded after 3, 5 and 8 days.

Antagonistic activity test of *Trichoderma* and *B. subtilis* against *P. drechsleri* under field conditions: To confirm the laboratory results of the antagonistic ability of *Trichoderma* isolates and *Bacillus subtilis* against root rot of muskmelon, field trails were conducted during two growing seasons of muskmelon. For mass production, antagonistic and pathogenic fungi were cultured on PDA medium and incubated at 25 Cº for 10 days and conidia were harvested from the cultured plate with the help of sapatula. The wheat bran (about 250 g) was double autoclaved at 121 Cº for 20 minutes and then cooled at room temperature. Conidial suspentions of 2×10^8/ml were prepared using hemocytometer, mixed in wheat bran and incubated for 10 days in dark.

Bacterial biocontrol agent, *Bacillus subtilis* was cultured on nutrient broth for three days and then bacterial cells were harvested by centrifugation process. Bacterial isolate was suspended in distilled water and concentration was adjusted at 1×10^9/ml and mixed in 250 g autoclaved wheat bran (Etebarian, 2006) check the combinatorial effects, all the tested antagonists (T_o) were mixed and applied to the soil.

Wheat bran having Trichoderma isolates and *Bacillus subtilis* were applied to the soil just before three days of sowing, while pathogenic fungi were applied to the soil after one day. The experiment was done at the research area of the College of Agriculture, University of Sargodha for two consecutive growing seasons. The plot size was 11.25 by18 m and the plant to plant and row to row distance was 0.40 m and 1 m respectively. Each set of experiment was repeated three times and twenty-five plants were used in each replication.

Percent disease incidence and survival assessment: Data of infected plants were recorded 45 days of the sowing and percent disease incidence and plant survival rate were calculated by the following formulas:

Formula 2.

$$\text{Perccent disease incidence} = \frac{number\ of\ infected\ plants}{Total\ number\ of\ plants} \times 100$$

Formula 3.

$$\text{Percent survival} = \frac{Total\ number\ of\ plants - infected\ plants}{Total\ number\ of\ plants} \times 100$$

The data for dry weights of roots and shoot were recorded after 45 days at experiment completion.

Statistical analysis:

The data of inhibition percentage, percent disease incidence and plant survival rate were analyzed by two-factor factorial analyses to check the significance of treatments at different time interval. Least significant difference (LSD) was used for treatments mean separation. All statistical analysis was carried out using R.3.0.3-Statistical package.

Results

Inhibition of pathogen in Dual culture: All tested bio-control agents showed significant mycelial inhibition against *P. drechsleri* in all dual culture methods, however, the best inhibition was provided by *T. asperellum* TH. Overall, the following trend of treatments; *T. asperellum* TH >*T. harzianum* HK >*T. harzianum* HM >*Bacillus* subtilis was observed in respect to their efficacy against root rot pathogen. Efficacy of biocontrol agents was higher in dual culture II as compared to the other two methods because *P. drechsleri* was inhibited by higher inoculum of both *Trichoderma* isolates and *B. subtilis* (Table 1).

Suppression of pathogen by fungal Metabolites: Non-volatile metabolites of all treatments significantly ($p<0.01$) inhibited the mycelial growth of *P. drechsleri*. *Trichoderma asperellum* TH showed maximum (46.8%) mycelial growth inhibition, while *B. subtilis* showed a minimum inhibition rate of 26.6% (Table 2).

In case of volatile metabolites, *T. asperellum* gave the highest mycelial growth inhibition (35.02%) among tested isolates, while *T. harzianum* HK, *T. harzianum* HM and *B. subtilis* gave 19.5%, 16.0% and 15.3% inhibition respectively (Table 2). Among volatile and non-volatile metabolites, non-volatile metabolites represent high inhibition potential against the pathogen.

Antagonistic activity test of *Trichoderma* isolates and *B. subtilis* against *P. drechsleri* under field conditions: Results of both growing seasons confirmed that all the tested biocontrol agents highly affected the incidence and survival rate of muskmelon plants as compared to control treatments. There was a significant variation of treatment on percent disease incidence ($F_{5,\ 12}$ = 189, $p<0.001$), percent plant survival rate ($F_{5,\ 12}$ = 189, $p<0.001$), dry root ($F_{5,\ 12}$ = 70.4, $p<0.001$) and dry shoot weight ($F_{5,\ 12}$ = 289, $p<0.001$) during first growing season. Joint action of all treatments (T_o) showed minimum percent disease incidence (20%) as compared to other tested treatments. The survival rate of plants (80%) and dry root and shoot weight was higher in rows inoculated with the T_o treatment (Fig. 1).

The decreasing trend of percent disease incidence was observed during second growing season while the percent survival rate was increased compared to first growing season. During second growing season, the treatment effect on percent disease incidence ($F_{5,\ 12}$ = 218, $p<0.001$), percent plant survival rate ($F_{5,\ 12}$ = 218, $p<0.001$), dry root weight ($F_{5,\ 12}$ = 63.5, $p<0.001$) and dry shoot weight ($F_{5,\ 12}$ = 416, $p<0.001$) was highly significant (Fig. 2).

Table 1. Effect of different biocontrol agents on percent inhibition growth of *Phytophthora drechsleri* at different time intervals by dual culture techniques.

Treatments	Dual culture # 1			Dual culture # 2			Dual culture # 3		
	3rd day	5th day	8th day	3rd day	5th day	8th day	3rd day	5th day	8th day
T. asperellum TH	22.69±1.42^{a}	47.31±0.51^{a}	64.33±0.38^{a}	28.09±0.54^{a}	58.03± 0.51^{a}	72.12±0.49^{a}	19.70±2.59^{a}	50.29±1.07^{a}	66.66±0.56^{a}
T. harzianum HK	15.12±1.95^{bc}	41.06±0.51^{b}	58.94±0.37^{b}	23.23±1.94^{a}	55.65± 0.30^{b}	69.86±0.49^{ab}	15.12±1.95^{ab}	46.12±1.07^{ab}	62.89±0.81^{b}
T. harzianum HM	11.87± 1.43^{c}	39.88±1.19^{b}	52.72±0.81^{c}	16.20±1.43^{b}	49.10±0.51^{d}	63.84±0.65^{c}	10.79±0.93^{b}	43.15±0.78^{bc}	55.17±0.49^{c}
Bacillus subtilis	17.17± 0.43^{b}	41.66±1.57^{b}	52.72±1.46^{c}	16.83±0.49^{b}	52.23±0.58^{c}	69.08±0.51^{b}	10.94±0.23^{b}	40.68±0.81^{c}	53.41±0.83^{c}
F value (3,8)	10.2	9.87	40.2	20.0	63.1	41.3	6.20	19.0	81.2
P value	<0.05	<0.05	<0.001	<0.001	<0.001	<0.001	<0.05	<0.001	<0.001

Means sharing similar letter with in a column are statistically non-significant ($p>0.05$)

Table 2. Effect of metabolites on percent inhibition growth of *Phytophthora drechsleri* at different time intervals.

Treatments	Volatile metabolites			Nonvolatile metabolites		
	3rd day	5th day	8th day	3rd day	5th day	8th day
T. asperellum TH	14.04±0.93^{a}	24.10±0.89^{a}	35.02±1.17^{a}	40.53±1.42^{a}	39.87±0.59^{a}	46.88±0.86^{a}
T. harzianum HK	11.33±1.95^{ab}	16.66±1.19^{b}	19.58±0.49^{b}	34.70±0.79^{b}	32.14±0.89^{b}	34.83±0.67^{b}
T. harzianum HM	7.53±0.93^{b}	9.81±1.36^{c}	16±0.67^{bc}	30.98±1.25^{bc}	25.29±1.07^{c}	29.94±0.65^{c}
Bacillus subtilis	7.86±0.42^{b}	10.69±0.72^{c}	15.38±0.49^{c}	27.23±0.28^{c}	21.46±0.72^{d}	26.69±0.32^{d}
F value	6.64	37.8	146	29.7	139.0	192
P	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001

Means sharing similar letter with in a column are statistically non-significant ($p>0.05$)

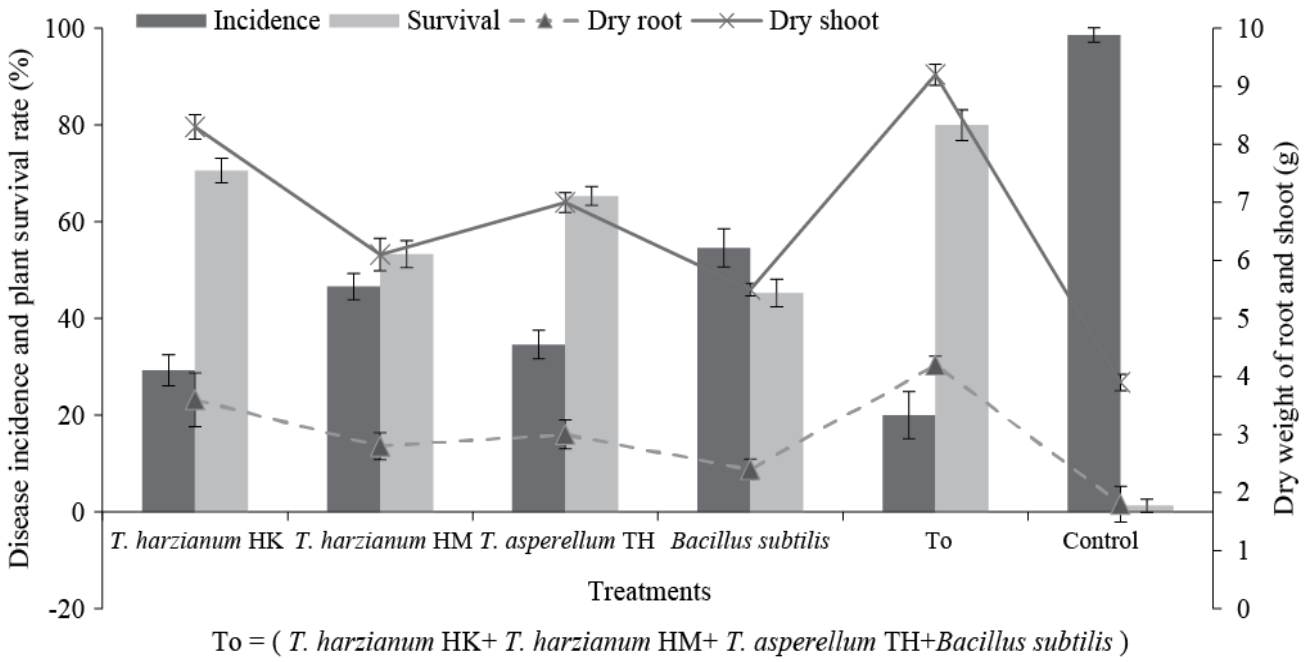

Fig. 1. Effect of different treatments on %disease incidence, % plant survival rate and dry root and shoot weight during first growing season after forty five days of application.

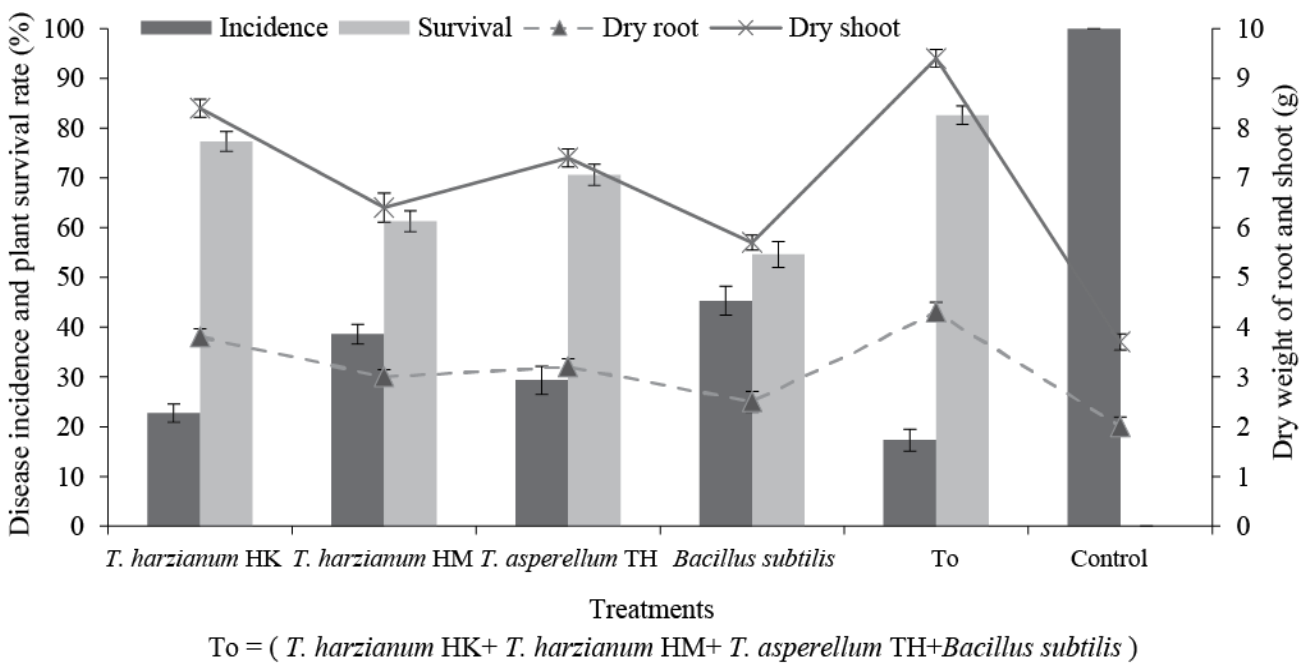

Fig. 2. Effect of different treatments on % disease incidence, % plant survival rate and dry root and shoot weight during second growing season after forty five days of application.

Discussion

Many soil born fungi are inhibited by different isolates of *Trichoderma* (Chet, 1987; Heidari *et al.,* 2005). Our results confirmed that *Trichoderma* isolates had great potential to inhibit the growth of *P. drechsleri*. Maximum percent inhibition of tested pathogen was shown by *T. asperellum.* Non-volatile metabolites produced by *T. asperellum* were also effective as compared to other fungi. *Trichoderma* isolates produced many nonvolatile and volatile metabolites like tricholin, peptaibols, massoilactone, gliovirin, heptelidic acid, alamethicins, harianic acid, glisoprenins and others which are lethal to plant pathogens (Qualhato *et al.,* 2013), so inhibition of our tested pathogen may be due to such metabolites produced by Trichoderma species as described above. In case of nonvolatile metabolites, *T. asperellum* showed 46.88% while volatile metabolites gave 35.02% mycelial growth inhibition against root rot pathogen. Tondje *et al.,* (2007) reported the antagonistic potential of *T. asperellum* against different plant pathogens which was similar to our present findings. Metabolites of *T. harzianum* have fungistatic

activity against *P. drechsleri* (Okhovvat, 1997). (Moayedi & Ghalamfarsa, 2011) reported the antagonistic potential of *T. harzianum* T39, *T. harzianum* M, *T. virens* and *T. viride* against *P. drechsleri* and also showed that nonvolatile and volatile metabolites of all *Trichoderma* isolates had inhibitory effect against pathogen. Their results were in agreement with the present investigation as volatile and nonvolatile metabolites of both *T. harzianum* isolates gave higher significant inhibition percentage.

The antifungal activity of *Bacillus* against different plant pathogens such as *P. drechsleri* and others have been reported by number of workers. Antifungal activity of Bacillus was due to proteinaceous substance working with other some substances (Wang *et al.,* 2013; Chung *et al.,* 2008). In present results Bacillus, isolate showed significant inhibition of *P. drechsleri*. In field experiment percent disease incidence and percent survival rate of plants, second season trial showed good results compared to the first season. Many researchers confirmed that the antagonistic fungi Trichoderma and Bacillus could survive for many years in soil (Longa *et al*., 2009; Setlow, 2014; Ulrich *et al*., 2018). So, our experiment confirmed that the population of antagonists was already present in next growing season in the soil because the incidence was minimum while survival rate was increased during second growing season. Hamed *et al.,* (2012) studied that incidence of root rot of beans which was 65 to 68%, decreased after the soil application of *T. harzianum*. Abdel-Kadir *et al.,* (2011) also reported that root rot incidence was decreased after the application of *T. harzianum* of faba plants. The suppression of soil borne pathogens after the application of biocontrol agents was due to antibiosis, competition or mycoparasitism mechanisms (Elad, 1996). Tested biocontrol agents increase the root and shoot weight of muskmelon plants. It was demonstrated that microorganisms had plant growth promoting ability and have significants effects on plant growth (Nagórska *et al.,* 2007; Naureen, 2009; Erdogan & Benlioglu, 2010). Overall, the present study provided insights into the utilization of microbial agents to manage the plant pathogenic *P. drechsleri* and potential replacement of hazardous chemical fungicides.

Conclusion

The present study demonstrated that *Trichoderma* and *Bacillus* species were potential biocontrol agents that showed significant levels of antifungal ability against *P. drechsleri*. The pathogen was suppressed in both laboratory and field experiments by biocontrol fungi and bacteria. The results also showed that tested *Trichoderma* and *Bacillus* isolates have potential to inhibit the mycelial growth of pathogen at low inoculum level. These biocontrol agents also have plant growth promoting ability. The utilization of biocontrol agents to control fungal diseases is opening new insights to replace the health hazardous synthetic chemicals. The results suggested the use of these environmental friendly methods to develop a healthy society and resolve the food security issues.

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