Environment, Biodiversity & Soil Security (EBSS)
http://jenvbs.journals.ekb.eg//

Cellulolytic Activity of *Trichoderma reesei* and *Bacillus subtilis* Against the Plant Pathogen *Pythiumdebaryanum*

A.A. Salem and H.M. Abdel-Rahman*
Agricultural Microbiology Dept., Faculty Agriculture, Moshtohor, Benha Univ., Egypt.

THIS work focused on the degradability of *Pythium debaryanum* cell wall by cellulase enzymes produced by *Bacillus subtilis* MK537378 and *Trichoderma reesei* MK934489.*B. subtilis* showed higher Exo-1,4-*β*-D glucanase and Endo-1,4-*β* D-glucanase activities than *T. reesei* while the highest β-glucosidase activities were recorded for *T. reesei*. Antagonistic activities against *P. debaryanum* were investigated by the tested *B. subtilis* and *T. reesei*. Moreover, the cellulolytic activity of both *B. subtilis* and *T. reesei* against *P. debaryanum* was confirmed by using Congo red staining technique. Microscopic observations showed clear hyphal lysis and degradation of the fungal cell wall in a dual plate assay. Results of the greenhouse experiment emphasized that the inoculated tomato seeds with *B. subtilis* or *T. reesei* in the presence of *P. debaryanum* showed 73.5% and 76% reduction in disease incidence, respectively as compared to the seed treated with pathogen alone (85%). Dehydrogenase activity (DHA) of soil rhizosphere was significantly increased in all inoculated soil with *B. subtilis* and/or *T. reesei* compared with uninoculated ones. The inoculation of tomato with *B. subtilis* and *T. reesei* in presence of *P. debaryanum* induced high activities of peroxidase, polyphenoloxidase, and chitinase by increasing 49.3, 55.2, and 56.9%, respectively, over the control. The grow thparameter sand yield of thetoma to plants significantly increased in response to the inoculation of *B. subtilis* MK537378 and *T. reesei* MK934489 compared to individual inoculation.

Keywords: Cellulase-producing bacteria, Biocontrol, *Pythiumdebaryanum,* Defense enzymes, and Tomato yield .

Introduction

Tomato (*Lycopersicon esculentum* L.) is considered to be a major vegetable crop grown worldwide. In Egypt, tomato cultivation covers about 32% of the total vegetable-growing area; with total production being approximately 16% of total vegetable production (Elshahawy et al., 2018; Abd-Elgawad 2020). There is little information available about the possibility of *Pythium* spp. inciting damping-off and root rot diseases on tomato in Egypt (Elshahawy et al. 2018). A major threat to agriculture is soil-borne diseases which extensively decline the crop yield. The genus *Pythium* contains many species that are important because of the economic losses that result from a reduction in yield associated with their diseases. *Pythium* spp. usually cause pre- and post-emergence damping-off, a disturbing agricultural disease. *Pythium*is a soil-borne pathogen that produces sporangiospores and the life cycle occurs within the soil. The pathogen infects plants primarily through the root system. (Zitnick-Anderson et al. 2017). Synthetic pesticidesaremainly a method for controlling damping-off (Muriungi et al. 2014). Concerns about environmental pollution associated with the use of synthetic pesticides in crop productionhave increased the need for alternative control methods (Rosenzweig et al. 2001).

*Corresponding author: E-mail: hany.abdelrahman@fagr.bu.edu.eg
Received: 5/4/2021; Accepted: 9/5/2021
DOI: 10.21608/jenvbs.2021.71242.1132

Biological control agents have been effectively used in controlling various diseases caused by *Pythium* spp. (Kipngeno et al. 2015 and El-Feky et al. 2019). Accordingly, *Trichoderma* is known to produce secondary metabolites and enzymes that attack or restrict the growth of other phytopathogenic fungi. Due to these properties, researchers agricultural field focused their attention on it to use in the biological control field (Taha et al. 2020). Moreover, rhizosphere bacteria are wonderful agents to control soil-borne plant pathogens. Many species of bacteria such as *Bacillus*, *Pseudomonas*, *Serratia* and *Arthrobacter* have been used in controlling fungal diseases (Joseph et al., 2007; Ashwini and Srividya 2014; Ghazi et al. 2018). Non-pathogenic soil *Bacillus* species offer several advantages over other organisms as they form endospores and hence can tolerate extreme pH, temperature and osmotic conditions. *Bacillus* species can colonize the root surface, increase plant growth and cause the lysis of fungal mycelia (Turner and Backman 1991 and Podile and Prakash, 1996).

Cellulases and related enzymes from certain bacteria and fungi are capable of degrading the cell wall of plant pathogens and controlling the plant disease (Saadia et al. 2008 andVinale et al. 2008). It has been reported that the β-1,3- and β-1,6-glucanases from *T. harzianum* hydrolyzed filamentous fungal cell walls, inhibited the growth of fungi tested, and reduced the disease incidence by *Pythium*. (Bruce et al. 1995 and Kumar et al. 2014). Thus, a combination of microbial strains and their enzymes could be useful as biocontrol agents to protect the seeds and plants from plant pathogens. Many cellulolytic fungi including *Trichoderma* sp, *Chaetomium* sp, and cellulolytic bcateria such as *Bacillus* sp play vital role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system as well as increased crop yields (Harman and Bjorkman, 1998 and Bae et al, 2009; Ashwini and Srividya, 2014).

The objectives of this current study were to investigate and confirm the role of cellulases enzymes produced by *Trichoderma reesei* MK934489 and *Bacillus subtilis* MK537378 for degrading the cell wall of *P.debaryanum*. Accordingly, using them for controlling *Pythium* disease in tomato.

Materials and Methods

Microorganisms

Bacillus subtilis MK537378 and *Trichoderma reesei* MK934489 strains were obtained from Agric. Microbiology Dept., Fac. of Agric., BenhaUniv., Egypt. The fungus *P.debaryanum* was obtained from Plant Pathology Dept., Fac. of Agric., BenhaUniv., Egypt. The originalbacterial and fungal cultures were maintained onnutrient agar and potato dextrose agar (PDA) slants, respectively. Maintenance media in the case of *B.subtilis* MK537378 and *T.reesei* MK934489 were supplied with 0.5% cellulose and stock cultures were keptat 5℃.

Fermentation and cellulase enzymes production

Modified Reese and Mandel's basal medium, (Salem and Abdel-Rahman, 2015) was used for cellulase enzyme production (Rice straw, 10; Proteose peptone, 0.25; $(NH_4)_2SO_4$,1.4; Urea, 0.3; KH_2PO_4, 2.0; $CaCl_2.2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3; (g L^{-1}) Tween 80, 1ml and 1 ml.L^{-1} of trace metal solution ($FeSO_4.7H_2O$, 5.0; $MnSO_4.7H_2O$, 5.6; $ZnSO_4.7H_2O$, 3.3 and $CoCl_2.2\ H_2O$, 2.0 (mg L^{-1}). About 95 mL of the production medium were dispended into 250 mLErlenmeyerflasks, sterilized and inoculated with 5 ml of a 2-days-old and 5-days-old inoculum of *B.subtilis* MK537378 and *T.reesei* MK934489, respectively. The inoculated flasks were incubated with shaking at 150 rpm and 30°C for 3 days. The cultureswere centrifuged at 4000 rpm for 30 min at 4°C. Thesupernatant was used forthe measurement of cellulase activity.

Cellulase enzymes determination

Endo-1,4-β-D-glucanase (CMCase) and Exo-1,4-β-D-glucanase activity were assayed using a modified method described by Wood and Bhat (1998). Cellobiase activity was determined by a modification of the method described by Berghem and Petterson (1974). The released glucose was determined using dinitrosalicylic acid method.

Antagonistic activity

B.subtilis MK537378 and *T.reesei* MK934489 were screened on PDA medium (containing 1% CMC) for their ability to inhibit the growth of *P. debaryanum* in dual Petri dishes culture test as described by Hariprasad and Niranjana (2009). The previous bio-agents were tested against the pathogenic fungi using dual culture technique. Briefly, the bacterialand fungal strains were seeded at the edges of 90 mm Petri plates containing PDA and incubated for 36 hr at 28±2°C. Then, afive-mm diameter disk of pathogenic fungus was placed on the sub-terminal of the another plate edge. Plates were incubated at 28±2°C for 4-7 days. After incubation, the plates were examined for clear zone using congo red staining technique (Hendricks et al., 1995)

Biocontrol agents of pathogen and the germination of tomato seeds

For determining the effect of *B. subtilis* MK537378 or *T. reesei* MK934489 in dividually

or in presence of *P. debaryanum* on tomato seeds germination, the method of Bákonyi et al., (2013) was used. The spore's suspension (10^4/ml) of *T. reesei* MK934489 and *P. debaryanum* and cell suspension (10^8/ml) of *B. subtilis* MK537378 were used by rate 35 µl per seed.

Biocontrol agent and pathogen inoculum preparation

The inocula of *T. reesei* MK934489 and *P. debaryanum* were prepared by standard procedures according to Rini and Sulochana, (2007) using a one-liter jar containing sorghum seeds, sand and water medium. After sterilization, the medium was inoculated with the mycelial disc of *T. reesei* MK934489 or *P. debaryanum* and was incubated for two weeks at 25°C. However, the inoculum of *B. subtilis* MK537378 was prepared by growing the bacterial strain in the nutrient broth medium at 30 °C with continuous shaking (140 rpm) up to optimum growth. The cells were harvested by centrifugation (4000 rpm) at 4 for 10 min. The cells were suspended using a physiological salt solution.

Seed coating

Before seeding, tomato seeds (approximately 6 g) were surface sterilized with 1% Ca $(OCl)_2$ for 3 min, washed thoroughly in sterilized water. Three grams of the wet seeds were mixed by stirring with 5ml of either *B. subtilis* MK537378 cell suspension and /or *T. reesei* MK934489 sporessuspension. Then, the coated seeds were spread on plastic trays and stored at 25°C away from direct sunlight to dry for 2 hr.

Efficacy of B. subtilis MK537378 and T. reesei MK934489against P. debaryanum

In 2020, a greenhouse experiment was conducted at the Faculty of Agriculture Experiment Station, Fac. of Agric., Benha Univ., Egypt to study the efficacy of biocontrol agents, i.e., *B. subtilis* MK537378 and *T. reesei* MK934489 individually or in their combined mixture, against damping-off disease of tomato caused by *P. debaryanum* were evaluated under greenhouse conditions. Sterilized plastic pots (30 cm diameter) were filled with10 kg sterilized soil (Table 1) that was mixed with *P. debaryanum* inoculum at a rate of 3%. Potsand soil were sterilized by using 5% formaldehyde solution then left to dry to remove formaldehyde two weeks before use. Infested pots were irrigated for seven days before sowing. Ten tomato seeds (cv. 'strain B') were sown in each pot and five replicate pots were itemized for each treatment in a completely randomized experimental design. Befor esowing, Three-quarters of tomato seeds were coated with either *B. subtilis* MK537378 and/or *T. reesei* MK934489 suspensions. Subsequently, each pot was drenched with 5 ml of *B. subtilis* MK537378 inoculum (10^8 CFU.ml^{-1}) or 5 g of *T. reesei* MK934489 inoculum (10^5 spores. g^{-1}) individually or in their combined mixture. the boost additions of bio-agent strains were added, 30 and 50 days after sowing (DAS). The experiment included the following treatments: non-infested soil (control), soil infested with *P. debaryanum* only, *P. debaryanum* + T. *reesei*, *P. debaryanum* + *B. subtilis*, and *P. debaryanum* + *T. reesei* + *B. subtilis*. In two equal doses, nitrogen, phosphorus, and potassium were added at a rate of 3, 4, and 3 $gpot^{-1}$ as ammonium sulphate (20.5% N), calcium superphosphate (15.5 % P_2O_5), and potassium sulphate (48% K_2O), respectively. Pots were kept under greenhouse conditions until the end of the experiment.

Disease assessment

The percentage of pre- and post-emergence damping-off and the percentage of survival plants were recorded at 30DAS asdescribed by (Elshahawy and El-Mohamedy, 2019).

Determination of dehydrogenase activity

Dehydrogenase activity was determined periodically at initial, 30, 60 and 90 DAS in soil samples from the tomato rhizosphere using methods described by Schinner et al. (1996).

Determination of the chlorophyll content

The effect of the treatments on the chlorophyll contents in the tomato leaves was determined at75 DAS. Five leaf disks were collected from tomato leaves (five leaves from every plant and five plants per replicate of every treatment). Chlorophyll was extracted from tomato leaf disks according to the method described by (Fadeel 1962) and was determined spectro-photometrically (SCO-Tech, SPUV-19, Germany) using the wavelength 662, and 644 nm for chlorophyll a, and b, respectively. The pigments (as mg/g fresh weight) were calculated using the formula adapted by Wettestein (1957).

Determination of the defense enzymes

Peroxidase, polyphenoloxidase, and chitinase in tomato were estimated at 75 DAS according to the method described by Lee (1973), Bashan et al. (1985), and Monreal and Reese (1969), respectively. Peroxidase activity was expressed as the increase in absorbance at 470 nm/g fresh weight/ min. Polyphenoloxidase activity was expressed as the increase in absorbance at 475 nm/g fresh weight/minute. Chitinase activity was expressed as mM N-acetyl glucose amine equivalent released / gram fresh weight /60 min at 540 nm.

Plant growth and yield assessment

At the end of the growing season (110 DAS),

average of plant height, number of branches, plant dry weight, plant yield and estimated yield per feddan were calculated.

Statistical analysis

Analysis of variance (ANOVA) was performed using CoStat version 6.400 (CoHort software, Monterey, CA, 93940, USA). Mean values among treatments were compared by the Duncan test at 5% level (p = 0.05) of significance and presented as the mean values ± standard deviation (SD).

Results and Discussion

Cellulase enzymes activity

Regrading cellulase three components i.e., Endo-1,4-β D-glucanase [EC.3.2.1.4], Exo-1,4-β-Dglucanase [EC.3.2.1.91] and β-glucosidase [EC. 3.2.1.21], their activities were detected (Table 2) for both *B. subtilis* MK537378 and *T. reesei* MK934489. *B. subtilis* MK537378 showed higher Exo-1,4- β-D glucanase and Endo-1,4-β D-glucanase activity than *T. reesei* MK934489. However, the highest β-glucosidase activities were recorded for *T. reesei* MK934489. Many reports confirmed the ability of *Trichoderma* spices and *Bacillus subtilis* to produce large amount of cellulases for examples (Montero et al. 2007 and Ihrmark et al., 2010) on *Trichoderma* sp. and Ashwini & Srividya, (2014) on *Bacillus subtilis*.

Inhibitory effect of P. debaryanum by B. subtilis MK537378 and T. reesei MK934489

Since *B. subtilis* MK537378 and *T. reesei* MK934489 showed high potent to produce cellulase enzymes, it was of interest to investigate (*in vitro*) their antagonistic activity against *P. debaryanum*. Data illustrated in (Fig. 1) showed antagonistic activity of *B. subtilis* MK537378 and *T. reesei* MK934489 against *P. debaryanum*.

Confirming of cellulolytic activity by B.subtilis MK537378 and T.reesei MK934489 against P.debaryanum

Cellulolytic activity of both *B. subtilis* MK537378 and *T.reesei* MK934489 against *P. debaryanum* was confirmed by using Congo red staining technique. Plates that showed antagonistic activity were stained with Congo red dye for thirty minutes and then carefully washed by using sodium chloride solution several times. Data illustrated in Fig. 2 are showing zones of clearance in where *B. subtilis* MK537378 and *T. reesei* MK934489 were grown indicating cellulolytic activity.

On the other hand, places, where *P. debaryanum* grew, maintained the red color of Congo red stain that could be good evidence that antagonistic effect could be due to the cellulolytic activities of *B. subtilis* MK537378 and *T. reesei* MK934489. In this respect, many studies have been conducted confirming the role of cellulase in phytopathogen controlling. Saadia et al. (2008) reported that cellulase and xylanase are the major type of enzymes that contributed to the degradation of the cell wall of the phytopathogens. Woo & Lorito (2007) and Vinale et al. (2008) confirmed that in the interactions of *Trichoderma* with plants, different kinds of metabolites may act as inducers of resistance. These metabolites are usually proteins including enzymes viz., cellulases, xylanases, chitinases, and glucanases. So, *Trichoderma* spp. was able to lyse and destroy conidiophores and spores of pathogen. These effects may be related to enzymes secreted such as cellulase, α-glucanase, and others (Harman 2006). Similar processes were reported against *Pythium oligandrum*, *Phytophthora megasperma*, and *Pythium ultimum* (Benhamou et al., 1999).

TABLE 1. Particle size distribution and chemical analyses of soil

Sand (%)	Silt (%)	Clay (%)	Texture class	Organic matter (gKg^{-1})	Bulk density (gcm^{-3})	pH	EC (dSm^{-1})
22.5	31	46.5	clay	30.1	1.36	8.22	1.92
Soluble Cation and Anions (cmol kg^{-1} soil)							
Ca^{++}	Mg^{++}	Na^{+}	K^{+}	Cl^{-}	HCO_3^{-}	CO_3^{--}	SO_4^{--}
3.43	1.73	3.95	0.98	3.93	4.14	-	1.93

TABLE 2. Cellulase enzymes activity of *B. subtilis* MK537378 and *T. reesei* MK934489

Microorganisms	Cellulases activity (U/mL)		
	Endo-1,4-β D-glucanase	Exo-1,4- β-D-glucanase	β-glucosidase
Bacillus subtilis	2.9±0.020	1.8±0.010	0.7±0.002
Trichoderma reesei	2.8±0.030	1.5±0.040	1.1±0.005

Microscopical investigation on mycelial lysis of P.debaryanum

Since there was an antagonistic effect for *B. subtilis* MK537378 and *T. reesei* MK934489 against *P.debaryanum* and this antagonism could be due to the cellulolytic effect so it was of interest to investigate the lytic effect on mycelial growth of *P.debaryanum*-stained slides were microscopically investigated and theobtained results were illustrated in Fig. 3. As shown, it's clear that the mycelia of *P. debaryanum* were completely lysed at a lot of regions even the cytoplasm went out through the lysed regions.

The obtained results can be good and real evidence to say that the cellulolytic effect of *B. subtilis* MK537378 and *T. reesei* MK934489 is the most important reason for inhibition of *P. debaryanum* growth and development.

Similar results were obtained by Ashwini and Srividya (2014) who found that the fungal mycelium of *Colletotrichum gloeosporioides* OGC1 that grown with *B. subtilis* culture showed damage, swelling and distortions compared to the control that wasn't grown with any bacterial culture and did not appear these abnormal features. This indicates the mycolytic activity of the *Bacillus* culture against fungal pathogen. Similarly, Podile and Prakash (1996) reported that the lysis and dissolution of fungal mycelium of *Aspergillus niger* by *B. subtilis* AF1 strain were recorded.

Effects on seeds germination

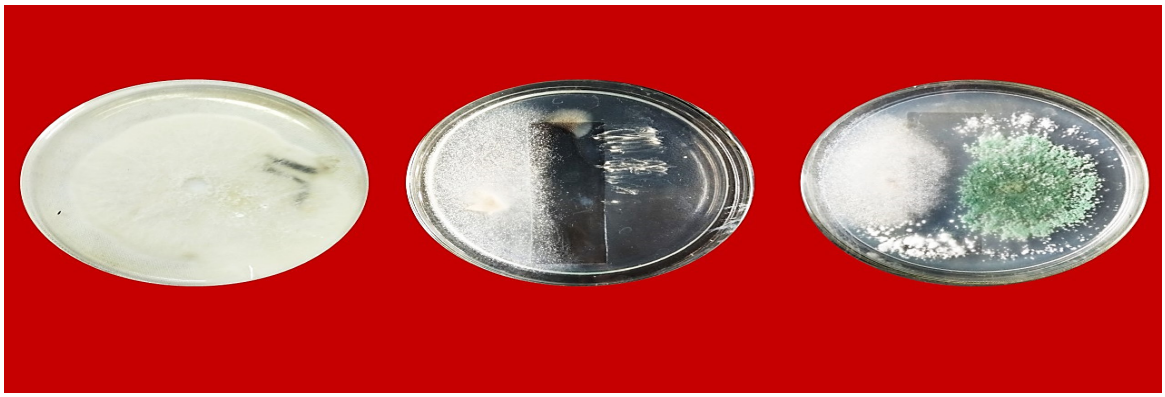
Data of Table 3 showed the treatments of the tomato seeds (Strain B) with *B.subtilis* MK537378 or *T. reesei*. the culture showed 5% and 1.5% increase in seeds germination respectively compared to the untreatedone (control). Moreover, the inoculation of the seed with *B. subtilis* MK537378 or *T. reesei* MK934489 with the pathogen showed 73.5% and 76% reduction in disease incidence, respectively as compared to the seed treated with pathogen alone (85%). This trend of results is in agreement with Ashwini and Srividya (2014) who reported that the chili seeds that treated with *Bacillus* sp. culture showed 100% germination index like to the untreated seeds and 65 % reduction in disease incidence by this treatment as compared to the seed treated with pathogen alone (77.5%). Moreover, Kamil et al., (2007) reported that the sunflower seeds that were coated with *B. licheniformis* recordeda high reduction in infection percentage of *R. solani* damping-off (from 25 to 60 %) as compared with the pathogen alone.

Effects on P. debaryanum infection under greenhouse conditions

Datain Table 4 show that soil infested with *P.debaryanum* significantly increased the incidence of damping-of foftomato seedlings and severely reduced the survival plant rate (11.7%) compared with thenon-infested control (93.3%). Potting soil that was treated with *B. subtilis* MK537378 or *T. reesei* significantly increased the survival rate compared with that infested with *P. debaryanum* solely, ranging between 72.1 - 79.2% (Table 4). However, higher survival rates of tomato seedlings were obtained inresponse to the combined inoculation of *B. subtilis* MK537378 and *T. reesei* MK934489 (83.0%). The previous results indicate that the treatment with *B. subtilis* MK537378 and *T. reesei*, individually or in combination, resulted inbestdiseaseman agement than the control. These results are in agreement with those obtained by Khare & Upadhyay (2009) and Kipngeno et al. (2015) who reported that The significantly lower percentage of pre-emergence damping-off of the seeds coated with either *B. subtilis* or *T.* spp under high disease pressure suggests that coating of seeds with *B. subtilis* or *T.* spp is effective against *P. aphanidermatum* damping-off.

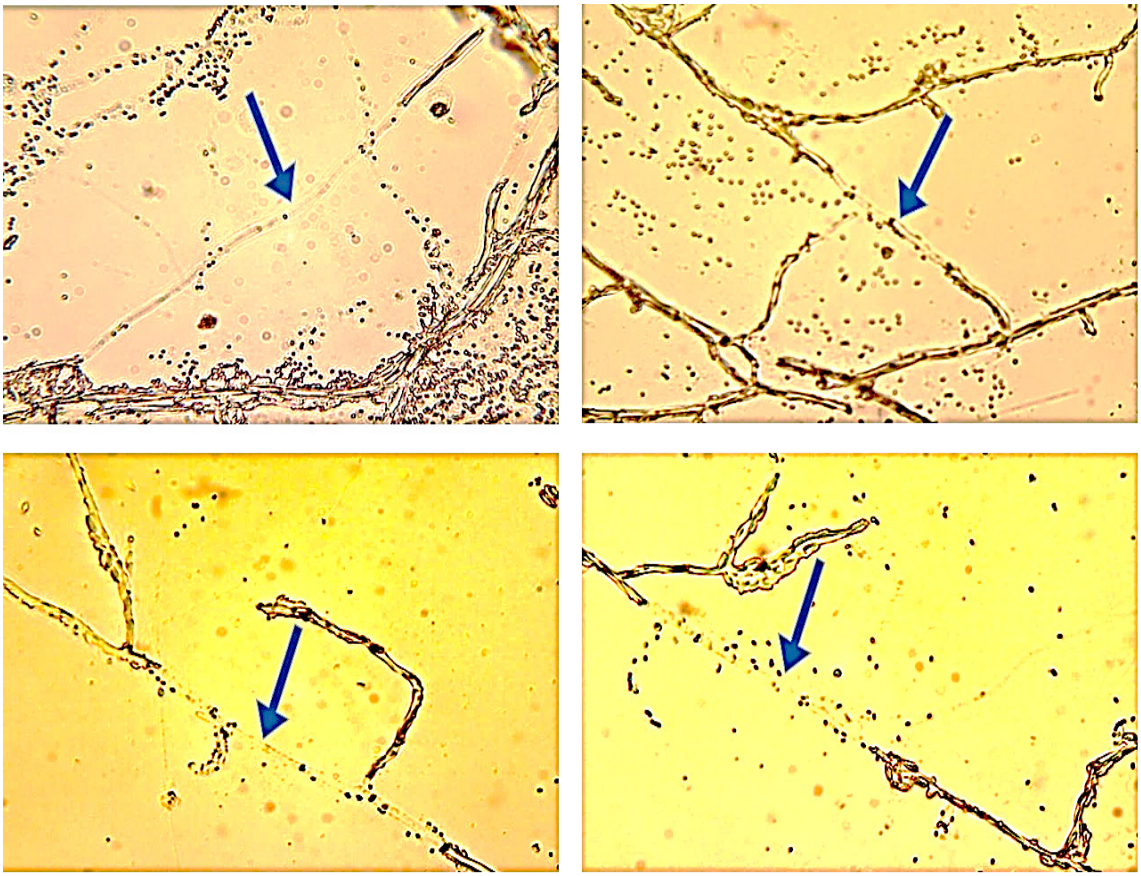
P.debaryanum. (control) | *B. subtilis* MK537378 against *P.debaryanum*. | *T. reesei* MK934489 against *P.debaryanum*.

Fig. 1. Antagonistic activity of *B. subtilis MK537378 and T. reesei MK934489 against P. debaryanum*



P.debaryanum. (control). | *B.subtilis* MK537378 against *P.debaryanum*. | *T.reesei* MK934489 against *P.debaryanum*.

Fig. 2. Confirming of cellulolytic activities of *B. subtilis* MK537378 and *T. reesei* MK934489 against *P.debaryanum* using Congo red staining technique



Dual growth of *B.subtilis* MK537378 and *P. debaryanum*

Dual growth of *T.reesei* MK934489 and *P. debaryanum*

Fig. 3. Microscopically investigation on mycelial lysis of *P. debaryanum*

The observations from this study indicate that seeds coated with *B. subtilis* MK537378 have cases of post-emergence damping-off less than those coated with *T. reesei*. This reveals a possibly higher efficacy of *B. subtilis* MK537378 in controlling *P. debaryanum* on tomato when compared to *T. reesei*. The variation could be due to the mechanism through which *B. subtilis* MK537378 or *T. reesei* MK934489 antagonize the pathogen as well as the ability of *B. subtilis* MK537378 to produce Endo-1,4-β-D-glucanase and Exo-1,4- β-D-glucanase by a higher amount than *T. reesei* MK934489 (Table 2). On the contrary, the treatment with the *B. subtilis* MK537378 was less effective in reducing the incidence of pre-emergence damping-off compared with individual treatment with *T. reesei*. The highest result was obtained by treating the soil with acombination of *B. subtilis* MK537378 and *T. reesei*, which reduced the incidence of pre- and post-emergence damping-off by 73.3% and 90.3%, respectively.

The inoculation of *B. subtilis* MK537378 and *T. reesei* MK934489 significantly decreased the percentage of infection and increased the percentage of survival plant scompared with the individual in oculations. The decreases in the infection percentage in case of inoculation with *B. subtilis* MK537378 and *T. reesei* MK934489 maybe due to the synergistic effect between two microorganisms. They can produce enzymes that attack the cell components of pathogens (Sivan et al., 1984 and Elad, 2000). In this study, microscopic observations showed clear hyphal lysis and degradation of the fungal cell wall in a dual plate assay. So, the suppressive effect expressed by the *B. subtilis* MK537378 and *T. reesei* MK934489 strains might be due to that mechanism. Previous reports showed that

microorganisms competent to degradecellulose, which is a major constituent of the fungal cell wall of oomycetes, play an important role in the biological control of fungal pathogens (Abdullah et al., 2008, Sharma, 2021 and Singh et al., 2021). However, a new mechanism has been added, namely, *Trichoderma*-induced resistance (Małolepsza et al., 2017) or *Bacillus*-induced resistance within the plant against fungal attack (Kloepper et al., 2004)

Effects on dehydrogenase activity

Dehydrogenase activity (DHA) was determined in the soil as a criterion for respiration rate and total microbial activity. Data in Table 5 show the values of dehydrogenase activity in the soil rhizosphere of tomato under-investigated treatments. Obtained data reveals significant increases in DHA values in all inoculated soil with *B. subtilis* MK537378 and/or *T. reesei* MK934489 compared with uninoculated ones. The highest values of DHA were revealed in tomato rhizosphere that treated with dual inoculated with *B. subtilis* MK537378 and *T. reesei* MK934489 in all determination periods. Higher values of DHA in dual application interpret the beneficial effect of inoculation with *B. subtilis* MK537378 and *T. reesei* MK934489 in proliferation and enhancement of microbial biomass in the rhizosphere (Abdel-Rahman, 2009).

By comparing the results of the dehydrogenase enzyme in the rhizosphere of the inoculated plants with *B. subtilis* MK537378 or *T. reesei* MK934489, it is found that DHA significantly increased in the case of *B. subtilis* MK53737 inoculation compared to inoculation with *T. reesei* MK934489. Higher records of DHA with *B. subtilis* MK537378 inoculation are likely to be due to the effective role of inoculation with previous strainfor enhancing colonization of introduced biocontrol strains for plant roots beside its role in enhancing the microbial community in the rhizosphere zone. Moreover, the inoculation might lead to the accumulation of available nutrients and thus stimulating the microorganisms in the rhizosphere. This was true in all experimental periods.

From the obtained data, it's noticed that the DHA in inoculated treatments with *B. subtilis* MK537378 and/or *T. reesei* MK934489 increased gradually from the first of the growing season up to 60 DAS and decreased again until 90 DAS. The periodic increase of DHA may be due to the boost addition of biocontrol agent strains. The highest value of DHA in this period was observed with dual inoculation of two biocontrol agents' strains followed by individual inoculation with *B. subtilis* MK537378 and *T. reesei* MK934489. It worth to stated that the increment of DHA records at 60 DAS may be due to the plants in this period enter the flowering stage where root exudates increase and enhance the number and activity of microorganisms in the rhizosphere (shams et al, 2013 and Abdel-Rahman et al, 2017).

Effects on chlorophyll contents in tomato

Leafchlorophyllcontentoftomatosignificantly increased in the treatments that inoculated with *T. reesei* MK934489 and/or *B. subtilis* MK537378 compared with uninoculated ones. Inoculation of *T. reesei* MK934489 markedly increased the chlorophylla and chlorophyllb compared with the infested one. Data in the Table 7 showed that the treatment oftomato with *B. subtilis* MK537378 gave significant increases in the chlorophyll content intomato leaves by 3.49 and 4.07% respectively, compared to that inoculated with *T. reesei*. The present data are in accordance with those obtained by Gajera et al. (2013).

TABLE 3. Effect of seed treatment with *B. subtilis* MK537378 and *T. reesei* MK934489 on seed germination under lab. condition

Treatments	Germination %	Infection %
Uninoculated seeds	95±1.3b	0±0.0d
Seed with pathogen	7.5±1.1d	85±1.08a
Seed inoculated with *B. subtilis*	100±2.6a	0±0.0d
Seed inoculated with *T. reesei*	98.5±1.8a	0±0.0d
Seed inoculated with *B. subtilis* + Pathogen	88±1.4c	11.5±1.04b
Seed inoculated with *T. reesei*+ Pathogen	93±1.03b	9±0.43c

Means ± standard deviation with in a column followed by the same letter are not significantly different at P= 0.05 when compared by Duncan test

The improvement of chlorophyll contentmay helpin the development of the plant by making physiological operations work better through increasing the metabolic processes of carbon. On the other hand, the increment of chlorophyll content will be increasing the metabolismofcarbohydrates compounds by fixation of CO_2(Hopkins, 1999).

Inthis study, the increase inchlorophyll content of tomato leaves may besubject tonitrogena vailability for the plantby biocontrol agent strains. Hashem et al. (2017) reported that the inoculation with *B. subtilis* induced the chlorophyll pigments synthesis. The photosynthetic pigments such as chlorophyll-a and b were reduced in uninoculated one (control). However, the inoculation of *B. subtilis* stimulates an increase of total chlorophyll by a rate 33.61%. Additionally, Al-Ezerjawi and Kadhim (2014) stated that *T. harzianum* might have colonizedaround theroot, increased therootbiomass and increased the availability of nutrients as well as interacted with the plant for exchangemetabolitesand that could cause significant changesin plant metabolism.

Defense enzymes in tomato

The data in Table 6 showed that there was a markable increase in the induction of defense enzymes (peroxidase, polyphenoloxidase, and chitinase) in the treated tomato as compared to the control. The inoculation of *B. subtilis* MK537378 and *T. reesei* MK934489 in presence of *P. debaryanum* induced high activities of peroxidase, polyphenoloxidase, and chitinase by increasing 49.3, 55.2 and 56.9%, respectively, over the control. Microbial-induced resistance, a novel mechanism that explains the ability of biocontrol agents to suppress phytopathogens in plants, is recently known. These results are similar to those of Li et al. (2008) who reported that the inoculation with *B. subtilis* AR12 induced systemic resistance against tomato pathogen. The production of antioxidant enzymes in the plant was increased significantly after *B. subtilis* AR12 induced compared to the controls.

TABLE 4. Effect of inoculation tomato with *B. subtilis* MK537378 and *T. reesei* MK934489 on damping-off and disease incidence in soil infested with *P. debaryanum*

Treatment	Damping-off and survival plants		
	% pre-emergence	% post-emergence	% survival plants
Non-infested soil (control)	6.67±0.58d	0.00±0.0b	93.3±0.6a
Soil infested with *P. debaryanum*	50.00±2.5a	38.33±2.6a	11.7±1.9c
P. debaryanum + T. *reesei*	16.67±1.44b	4.17±0.8b	79.2±0.8b
P. debaryanum + *B. subtilis*	20.00±1.53b	7.87±1.9b	72.1±2.5b
P. debaryanum + *T. reesei* + *B. subtilis*	13.33±0.58c	3.70±0.7b	83.0±1.0ab

Means ± standard deviation with in a column followed by the same letter are not significantly different at P= 0.05 when compared by Duncan test

TABLE 5. Effect of the inoculation of *T. reesei* MK934489 and *B. subtilis* MK537378 on dehydrogenase activity intomat or hizosphere of soil infested with *P. debaryanum*

Treatment	Dehydrogenase activity			
	Initial	30	60	90 days
Non-infested soil (control)	24.10±1.67d	32.55±2.25d	37.14±2.57e	31.50±2.18d
Soil infested with *P. debaryanum*	29.62±2.05b	32.55±2.23d	33.48±2.31d	30.67±2.12e
P. debaryanum + T. *reesei*	29.42±2.03ab	34.01±2.35c	43.08v±2.98c	39.12±2.70c
P. debaryanum + *B. subtilis*	27.12±1.87c	38.70±2.68b	46.21±3.19b	40.47±2.80b
P. debaryanum + *T. reesei* + *B. subtilis*	29.83±2.06a	39.43±2.73a	48.30±3.34a	45.38±3.14a

Means ± standard deviation with in a column followed by the same letter are not significantly different at P= 0.05 when compared by Duncan test

Moreover, Chen et al. (2005) state that once the *Trichoderma* spp. colonized the roots, the plant defense responses can become systemic and protect the entire plant from a range of pathogens and diseases. *T. harzianum* strain T22 was not only able to promote seedling growth but also induced plant resistance. In the current study, the reduction in tomato root rot disease incidence due to *B. subtilis* MK537378 and *T. reesei* MK934489 application may be due to an increase in the defense-related enzymes such as peroxidase, polyphenoloxidase and chitinase beside the ability of those strains to produce cellulases that destroyed cell wall of the pathogen. The defense enzymes play an important role in induced resistance by incorporating toxic products into plant cell walls, which reduce fungal activity. For example, the oxidation of phenols to oxidized toxic products (quinine), incorporation of phenolic compounds, and lignification of plant cell walls (Małolepsza et al., 2017).

Peroxidase and polyphenoloxidase activity significantly increased in inoculated with *T. reesei* MK934489 than that inoculated with *B. subtilis* MK537378. However, tomato inoculated with *B. subtilis* MK537378 observed higher values of chitinase activity compared with that inoculated with *T. reesei* MK934489.

Effectsontomato growth parameters and yield

The data presented in Table 8 revealed low values of the grow thparameters and yield of tomato plants, i.e., plant height, number of branches, plant dry weight, yield perplant and yield perfed danininfested soil with *P. debaryanum* solely compared with the other treatments. The grow thparameters and yield of the tomato plants increased significantly inresponse to the inoculation of *B. subtilis* MK537378 and *T. reesei* MK934489 compared to individual inoculation (Fig. 4). The present data are inaccordance with those obtainedby Shams et al. (2013) who state that the dual inoculation with plant growth-promoting bacteria gavea significant increase in all growth parameters and yield on lettuce compared with an individual one. Moreover, Zaghloul et al. (2007), reported that the use of biocontrolagents solelyorin combination led toa significant increase in the number of tomatofruitsper plant, the weight offruits and the totalfruit yield.

Plant grow th e nhancement by *B. subtilis* MK537378 and *T. reesei* MK934489 isdone through different mechanisms such as secretion of plant grow thregulators (Hoitink et al. 2006; Vinale et al. 2008 and Hashem et al. 2017), phosphates,and micronutrient solubilization (Altomare et al. 1999), secretion of exogenousenzymes, siderophores (Jalal *et al*. 1987) and vitamins (Inbar et al. 1994), as well as indirectly with the control of themajor and minorrootinfestingpathogensinthe rhizosphere (Harman et al. 2004).

Depending on the data in Tables 5 and 7, it could be noticed that the effective role of inoculation with biocontrol agent on the enhancement of microbial community and plant chlorophyll as well as second arymetabolites such as auxin-like compounds that might be as on for the improved growth and increased yield. Besides, root colonization increases the growth of the entire plant and thus increases plant productivity and yields. Symbiotic association with microbes' rhizosphere and the plant helps to surmount biotic stress and improve nutrient uptake (Harman et al. 2004). Also, Hashem et al. (2017) reported that *B. subtilis* enhanced the uptake of some macro- and micronutrient which led to enhance the plant growth at disease conditions and might be due to the regulation of various metabolic pathways such as antioxidant system and chlorophyll synthesis.

Conclusion

From the obtained results it can be concluded that *B. subtilis* MK537378 and *T. reesei* MK934489 showed high potent to produce cellulase enzymes and antagonistic activity against *P. debaryanum*. Microscopic have been conducted to show the role of cellulose-degrading enzymes in controlling the fungus or not, and the results have shown the mycelia of *P. debaryanum* were completely lysed at a lot of regions even the cytoplasm went out through the lysed regions. Greenhouse study was conducted on tomato and the results confirmed that damping-off disease caused by *Pythium debaryanum* can be controlled by using the culture of cellulases enzymes producers *Bacillus subtilis* MK537378 and *Trichoderma reesei* MK934489. Moreover, markable increase in the induction of defense enzymes (peroxidase, polyphenoloxidase, and chitinase), growth parameters, and yield of tomato plants were observed. Considering these facts as well as other features of *B. subtilis* MK537378 and *T. reesei* MK934489 strains, further investigations are ongoing in our laboratories.

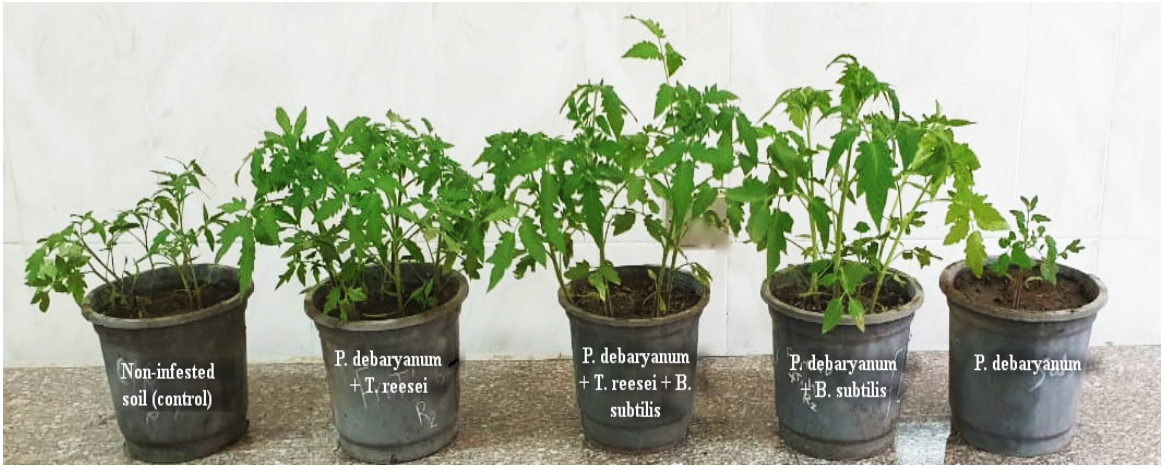


Fig. 4. Effect of inoculation with *B. subtilis* MK537378 and *T. reesei* MK934489 on tomato vegetative growth at 45DAS compared with infested and non-infested treatments

TABLE 6. Peroxidase, polyphenoloxidase and chitinase activities in tomato leave affected by the inoculation with *T.reesei* MK 934489 and *B.subtilis* MK537378 in presence of *P. debaryanum*

Treatment	Enzymeactivities		
	Peroxidase	Polyphenoloxidase	Chitinase
Non-infested soil (control)	0.224±0.02e	0.304±0.02e	0.736±0.05e
Soil infested with *P. debaryanum*	0.335±0.02d	0.490±0.03d	0.959±0.07d
P. debaryanum + *T. reesei*	0.406±0.03b	0.622±0.04b	1.317±0.09c
P. debaryanum + *B. subtilis*	0.349±0.02c	0.570±0.04c	1.403±0.10b
P. debaryanum + *T. reesei* + *B. subtilis*	0.442±0.03a	0.678±0.01a	1.709±0.02a

Means ± standard deviation with in a column followed by the same letter are not significantly different at P=0.05 when compared by Duncan test

TABLE 7. Effect of application of *T. reesei* MK934489 and *B. subtilis* MK537378 on chlorophyll content in tomato cultivated in soil infested with *P. debaryanum*

Treatment	Chlorophyll content (mg.g^{-1} fresh leaves)		
	Chlorophyll a	Chlorophyll b	Chlorophyll (a+b)
Non-infested soil (control)	2.30±0.12b	1.04±0.05b	3.34±0.18b
Soil infested with P. *debaryanum*	1.45±0.44c	0.72±0.22b	2.17±0.65c
P. debaryanum + *T. reesei*	3.32±0.72a	1.65±0.36a	4.97±1.08a
P. debaryanum + *B. subtilis*	3.44±0.71a	1.72±0.36a	5.16±1.07a
P. debaryanum + *T. reesei* + *B. subtilis*	3.87±0.56a	1.92±0.28a	5.79±0.84a

(a)Means ± standard deviation with in a column followed by the same letter are not significantly different at P= 0.05 when compared by Duncan test.

TABLE 8. Effect of application of *T.reesei* MK934489 and *B.subtilis* MK537378 on tomato growth parameters and yield.

Treatment	Plant height (cm)	No. of branches	Plant dry weight (g)	Yield/plant (kg)	Yield/fed. (metric ton)
Non-infested soil (control)	42.4±2.87c	3.8±0.45b	8.78±0.55c	0.80±0.07c	18.3±1.64c
Soil infested with *P. debaryanum*	33.0±3.29d	2.2±0.55c	5.62±0.61d	0.38±0.13d	8.7±3.00d
P. debaryanum + T. *reesei*	53.6±3.90b	5.6±0.45a	12.12±0.82b	1.32±0.09b	30.4±2.06b
P. debaryanum + *B. subtilis*	54.1±4.17b	5.8±0.45a	14.64±0.76a	1.38±0.08ab	31.7±1.92ab
P. debaryanum + *T. reesei* + *B. subtilis*	64.2±4.81a	6.2±0.45a	15.38±1.03a	1.44±0.08a	33.1±1.92a

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors declare their consent for publication.

Contribution of authors

This study was designed and implemented by all the authors, where all contributed to writing the manuscript, interpreting information presented and have read and agreed to the final version of the manuscript.

Funding

This research received no external funding.

Conflicts of Interest

The author declares no conflict of interest.

Acknowledgment

The authors would like to express sincere appreciation to all staffmembers of Agric. Microbiology Dept., Faculty of Agriculture at Moshtohor, Benha University, for their kind cooperation.

References

Abd-Elgawad MM (2020) Optimizing biological control agents for controlling nematodes of tomato in Egypt. *Egyptian Journal of Biological Pest Control,* 30, 1-10.

Abdel-Rahman HM, Salem AA, Moustafa MM, El-Garhy, HA (2017) A novice *Achromobacter* sp. EMCC1936 strain acts as a plant growth-promoting agent. *Acta Physiologiae Plantarum,* 39 (2), 61.

Abdelrahman HM (2009) Bio-organic farming efficiency on yield and quality of some medicinal plants (Doctoral dissertation, *Ph. D. Thesis,* Botany Department, Faculty of Agriculture, Moshtohor, Benha University).

Abdullah TM, Ali YN, Suleman P (2008) Biological control of Sclerotinia sclerotiorum (Lib.) de Bary with Trichoderma harzianum and Bacillus amyloliquefaciens. *Crop Prot.* **27**: 1354–1359.

Al-Ezerjawi NH, Kadhim JH (2014) Effect of Two Isolates of *Trichodermaharzianum* on Total Nitrogen, Chlorophyll a & b Contents and Yield of Wheat (*Triticum aestivum*. L) Class Eba'a-95.

Altomare C, Norvell WA, Björkman T, Harman GE (1999) Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichodermaharzianum* Rifai 1295-22. *Appl Environ Microbiol.* **65**:2926–2933.

Ashwini N, Srividya S (2014) Potentiality of Bacillus *subtilis* as biocontrol agent for management of anthracnose disease of chilli caused by *Colletotrichum gloeosporioides* OGC1. *3 Biotech,* **4** (2), 127-136.

Bae H, Sicher RC, Kim MS, Kim SH, Strem MD, Melnick RL, Bailey B (2009) The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in Theobroma cacao. *Journal of Experimental Botany,* **60** (11), 3279-3295.

Bákonyi N, Bott S, Gajdos E, Szabó A, Jakab A, Tóth B, Makleit P, Veres S (2013) Using Biofertilizer to Improve Seed Germination and Early Development of Maize. *Polish Journal of Environmental Studies,* **22** (6), 1595-1599.

Bashan Y, Okon Y, Henis Y (1985) Peroxidase, polyphenol oxidase, and phenols in relation to resistance against 214 Pseudomonas syringaepv. tomato in tomato plants. *Can J Bot.* **65**, 366–372.

Benhamou N, Rey K, Picard Y (1999) Ultraestructural and cyto chemical aspects of the interaction between the mycoparasite Pythium oligandrum and soil borne plant pathogens. *Phytopathology* **89**, 506–517.

Berghem LER and Petterson LG (1974) The mechanism of enzymatic cellulose degradation: Isolation and some properties of β-glucosidase from *Trichoderma koningii*. *Eur. J Biochem.* **46**, 295-305.

Björkman T, Blanchard LM, Harman GE (1998) Growth enhancement of shrunken-2 (sh2) sweet corn by *Trichoderma harzianum* 1295-22: effect of environmental stress. *Journal of the American Society for Horticultural Science,* **123** (1), 35-40.

Bruce A, Srinivasan U, Staines HJ, Highley TL (1995) Chitinase and laminarinase production in liquid culture by *Trichoderma* spp. and their role in biocontrol of wood decay fungi. *International Biodeterioration & Biodegradation,* **35** (4), 337-353.

Chen R, Harman GE, AfiocoMI, Cheng SG (2005) Proteins related to the biocontrol of Pythium damping-off in maize with *Trichoderma harzianum*. *J Integr Plant Biol.* **47** (8):988–997.

Elad Y (2000) Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Prot 19:709–714.

El-feky N, Essa T, Elzaawely AA, El-Zahaby HM (2019) Antagonistic activity of some bioagents against root rot diseases of pepper (*Capsicum annum* L.). *Environment, Biodiversity and Soil Security,* **3** (2019), 215-225.

Elshahawy I, Abouelnasr HM, Lashin SM, Darwesh OM (2018) First report of Pythium aphanidermatum infecting tomato in Egypt and its control using biogenic silver nanoparticles. *Journal of Plant Protection Research,* **58** (2).

Fadeel AA (1962) Location and properties of chloroplasts and pigment determination in roots. *Physiologia plantarum,* **15** (1), 130-146.

Ghazi AA, Attia EA, Rashed NM (2018) Management of charcoal rot (*Macrophominaphaseolina*) infection in geranium (*Pelargonium graveolens* L.) using biocontrol agents and essential oils. *Environment, Biodiversity and Soil Security,* **2** (2018), 131-142.

Hariprasad P, Niranjana SR (2009) Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato. *Plant and Soil*, **316** (1), 13-24.

Harman GE (2006) Overview of mechanisms and uses of Trichoderma spp. *Phytopathology* **96**, 190–194.

Harman GE, Bjorkman T. (1998) Potential and existing uses of Trichoderma and Gliocladium for plant disease control and plant growth enhancement. Trichoderma and Gliocladium: Enzymes, biological control and commercial applications. Vol. 2. London: Taylor & Francis Ltd,. pp. 229–65.

Hashem A, Abd-Allah EF, Alqarawi AA, Radhakrishnan R, Kumar A (2017) Plant defense approach of *Bacillus subtilis* (BERA71) against *Macrophominaphaseolina* (Tassi) Goid in mung bean. *Journal of Plant Interactions,* **12** (1), 390-401.

Hendricks CW, Doyle JD, Hugley B (1995) A new solid medium for enumerating cellulose-utilizing bacteria in soil. *Applied and Environmental Microbiology,* **61**(5), 2016-2019.

Hoitink HAJ, Madden LV, Dorrance AE (2006) Systemic resistance induced by Trichoderma spp. interactions between the host, the pathogen, the biocontrol agent, and soil organic matter quality. *Phytopathology* **96**,186–189.

Hopkins WG (1999) Introduction to plant physology. 2nd edn. John Wiley and Sons, Inc. USA

Ihrmark K, Asmail N, Ubhayasekera W, Melin P, Stenlid J, Karlsson M (2010) Comparative molecular evolution of Trichoderma chitinases in response to mycoparasitic interactions. *Evol Bioinform* **6**,1–26

Inbar J, Abramsky M, Cohen D, Chet I (1994) Plant growth enhancement and disease control by Trichoderma harzianum in vegetable seedlings growth under commercial conditions. *Eur J Plant Pathol.* **100**, 337–346

Jalal MAF, Love SK, Van der Helm D (1987) Siderophore mediated iron (III) uptake in Gliocladium virens (*Trichoderma virens*). 2. Role of ferric mono and dihydroxamates as iron transport agents. *J. InorganBiochem.* **29**:259–267.

Joseph B, Ranjan Patra R, Lawrence R. (2007) Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *International Journal of Plant Production,* **1** (2), 141-152.

Kamil Z, Rizk M, Saleh M, Moustafa S (2007) Isolation

and identification of rhizosphere soil chitinolytic bacteria and their potential in antifungal biocontrol. *Global J Mol Sci.* **2**, 57–66

Khare A, Upadhyay RS, (2009) Induction of mutant strains of *Trichoderma viride* 1433 for biocontrol of *Pythium aphanidermatum*. *Environ. Biol. Conserv.* **14**, 123–567.

Kipngeno P, Losenge T, Maina N, Kahangi E, Juma P (2015) Efficacy of Bacillus *subtilis* MK537378 and Trichoderma asperellum against *Pythium aphanidermatum* in tomatoes. *Biological Control,* **90**, 92-95.

Kloepper, J. W., Ryu, C. M., & Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology,* **94** (11), 1259-1266.

Kumar S, Roy PD, Lal M, Chand G, Singh V (2014) Mass multiplication and shelf life of Trichoderma species using various agro products. *Int Quar J Life Sci.* **9**:1143–1145

Lee TT (1973) On extraction and quantitation of plant peroxidase isoenzymes. Physiol Plant 29:198–203

Li SM, Hua GG, Liu HX, Guo JH (2008) Analysis of defence enzymes induced by antagonistic bacterium Bacillus *subtilis* MK537378 strain AR12 towards *Ralstonia solanacearum* in tomato. *Annals of Microbiology,* **58** (4), 573-578.

Małolepsza U, Nawrocka J, Szczech M (2017) *Trichoderma virens* 106 inoculation stimulates defence enzyme activities and enhances phenolic levels in tomato plants leading to lowered Rhizoctonia solani infection. *Biocontrol Sci Tech.* **27** (2):180–199.

Monreal J, Reese ET (1969) The chitinase of Serratia marcescens. *Can J Microbiol.* **15**, 689–696.

Moran R, Porath D (1980) Chlorophyll determination in intact tissues using N, N-dimethylformamide. *Plant Physiology,* **65** (3), 478-479.

Montero M, Sanz L, Rey M, Llobell A, Monte E (2007) Cloning and characterization of bgn16_3, coding for a β-1, 6-glucanase expressed during Trichoderma harzianum mycoparasitism. *J Appl Microbiol.* 103:1291–1300

Muriungi SJ, Mutitu EW, Muthomi JW (2014) Efficacy of cultural methods in the control of Rhizoctonia solani strains causing tomato damping-off in Kenya. *African Journal of Food, Agriculture, Nutrition and Development,* **14** (2).

Podile AR, Prakash AP (1996) Lysis and biological control of Aspergillus niger by Bacillus *subtilis* MK537378 AF 1. *Canadian Journal of Microbiology,* **42** (6), 533-538.

Rini CR, Sulochana KK (2007) Management of seedling rot of chili (*Capsicum annuum* L.) using *Trichoderma* spp. and fluorescent pseudomonads (*Pseudomonas fluorescens*). *Journal of Tropical Agriculture,* **44**, 79-82.

Rosenzweig C, EIglesias A, Yang XB, Epstein PR, Chivian E (2001) Climatechange and extreme weather events: implications for food production, plantdiseases, and pests. *Global Change Hum. Health* **2**, 90–104.

Saadia M, Ahmed S, Jamil A (2008) Isolation and cloning of cre1 gene from a filamentous fungus *Trichoderma harzianum*. *Pakistan Journal of Botany,* **40** (1), 421-426.

Salem AA, Abdel-Rahman HM (2015) Optimization and characterization of cellulolytic enzymes produced from *Gliocladiumroseum*. *Journal of Agricultural Chemistry and Biotechnology,* **6** (11), 473-488.

Schinner F, Oehlinger R, Kandeler E, Margesin R (1996) Methods in soil biology. Springer Lab Manuals. Part I:213–241.

Shams AS, Abdel-Rahman HM, El-Ramady HR (2013) Evaluation of integrated nutrient management practices for lettuce production under drip irrigation system. *Journal of Applied Sciences Research,* **9** (3), 2223-2231.

Sharma I. (2021) Phytopathogenic fungi and their biocontrol applications. In Fungi Bio-Prospects in Sustainable Agriculture, Environment and Nano-Technology (pp. 155-188). Academic Press.

Singh R, Upadhyay SK, Singh M, Sharma I, Sharma P, Kamboj P, Khan F (2021) Chitin, chitinases and chitin derivatives in biopharmaceutical, agricultural and environmental perspective. *Biointerface Res Appl Chem,* **11**(3), 9985-10005.

Sivan A, Elad Y, Chet I (1984) Biological control effects of new isolate of *Trichoderma harzianum* on *Phythiumaphanidermatum*. *Phytopathology* **74**:498–501.

Taha N, Kamel S, Elsakhawy T, Bayoumi Y, Omara AED, El-Ramady HR (2020) Sustainable Approaches of *Trichoderma* under Changing Environments for Vegetable Production.

Environment, Biodiversity and Soil Security, **4** (2020), 291-311.

Turner JT, Backman PA (1991) Factors relating to peanut yield increases following *Bacillussubtilis* seed treatment. *Plant Dis,* **75**, 347-353.

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M (2008) *Trichoderma*–plant–pathogen interactions. *Soil Biology and Biochemistry*, **40** (1), 1-10.

Wettestein D (1957) Chlorophyll-lethal under submicroscopic change in shape of the plastids. *Expt Cell Res.* **12**, 427-500.

Woo S, Lorito M (2007) Exploiting the interactions between fungal antagonists, pathogens and the plant for biocontro. In: Vurro M, Gressel J (Eds) Novel biotechnologies for biocontrol agent enhancement and management. Springer, Netherlands, pp 107–130.

Wood TM and Bhat KM (1998) Method for measuring cellulase activities. In Methods in Enzymology. Cellulose and Hemicellulose, Eds W.A. Wood and J.A. Kellogg, Vol. 160, pp. 87-112. Academic Press, New York

Zaghloul RA, Hanafy EA, Neweigy NA, & Khalifa NA (2007, March) Application of biofertilization and biological control for tomato production. In *12th Conference of Microbiology* (pp. 18-22).

Zitnick-Anderson KK, Norland JE, Luis E, Fortuna AM, Nelson BD (2017) Probability models based on soil properties for predicting presence-absence of *Pythium* in soybean roots. *Microbial Ecology,* **74** (3), 550-560.