TWO T*RICHODERMA* SPECIES AND *BACILLUS SUBTILIS* AS BIOCONTROL AGENTS AGAINST *RHIZOCTONIA* DISEASE AND THEIR INFLUENCE ON POTATO PRODUCTIVITY

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(Manuscript received 2 nd January 2017)

Abstract

Stem canker and black scurf caused by *Rhizoctonia solani* is a problem facing potato production. In this work, under greenhouse conditions, three compatible bioagents i.e., *Trichoderma koningii* and *T. harzianum* (in mixture) and *Bacillus subtilis* ATCC®11774™ were evaluated individually and in combinations for disease suppression and further effect on plant growth of potato plants. Radial growth of *R. solani* was inhibited by the two *Trichoderma* strains and *B. subtilis* in dual Petri plate assay. In two experiments, significant plant protection was achieved when either *B. subtilis* added to tubers or *Trichoderma* mixture added to the soil. However, soil application with *Trichoderma* either singly or in combination with tuber bacterization demonstrated the greatest suppression of cankers on potato plants. With respect to plant growth promotion, the greatest proportional increases in plant height were elicited by tuber bacterization combined with soil application of *Trichoderma* mixture. Dual tuber treatments by *Trichoderma* mixture with soil applications of bacteria led to the highest increase of plant stolons and leaf numbers in both experiments. Both combined applications and sole soil application by *T.* mixture recorded the same significant effect in increasing shoot fresh and dry weights of potato plants as well as improved tuber yield and some biochemical parameters (chlorophyll content, total phenol, peroxidase and polyphenoloxidase contents) significantly. This research suggests incorporation of such bioagents to suppress *Rhizoctonia* diseases and increase the productivity of potato.

Key words: *Trichoderma harzianum*, *Bacillus subtilis*, Bacterization with antagonist, potato yield, chlorophyll content, total phenols, peroxidase and polyphenoloxidase

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most important vegetable crops in Egypt, ranking fourth in yield production after rice, maize and wheat. In 2013, the cultivated area of potatoes in Egypt was 381379 Fadden, which produced 4265178 tons, while the annual Egypt exports reached 637,434 ton with a market value around $250 million (FAOSTAT © FAO, 2015). Stem canker and black scurf of potato is a serious disease commonly observed in most potato-producing areas of the world. The

disease is caused by specific anastomosis groups *Rhizoctonia solani* as AG-3 and AG-4. The teleomorph is *Thanatephorus cucumeris* [Frank] Donk), which is favored by the capacity of the fungus to survive in soil as sclerotia and mycelium in plant debris for long periods. *Rhizoctonia solani* AG-3 is relatively specific to potato. Dead tips of the developing sprouts and roots, cankers on underground stem parts and stolons, and sclerotia formation on progeny tubers resemble the typical black scurf symptoms. The formation of tuber-borne sclerotia downgrades tuber quality with the development of malformed tubers and an alteration in size and number of tubers. Disease severity is not always associated with yield reduction. (Banville *et al.*, 1996).

Cultural practices, solarization, chemical and biological control are the basic methods used for *R. solani* control. The development of fungicidal resistance, and the hazards on non-target organisms and environment, biological control represents the main concern of plant patholologists. However, current cultural and chemical control measures are not completely effective and *Rhizoctonia* disease has been remaining a persistent problem (Hicks *et al*., 2014).

Bacillus and *Pseudomonas* are the most investigated genera of the biocontrol agents *Bacillus subtilis* is among the most used biological agents against many soil phytopathogens including *R. solani* (Bhattacharjee and Dey, 2014). In this respect, Brewer and Larkin (2005) recorded that, among 28 tested potential biocontrol organisms, treatment with *B. subtilis* was most effective in reducing stem canker severity on potato (40-49% reduction). The antagonistic activity of *B. subtilis* may be attributed to the production of bioactive compounds and/or extracellular hydrolytic enzymes (Saber *et al*., 2015).

Several *Trichoderma* spp. are well documented as effective biological control agents against numerous plant pathogens, including *R. solani* (Hicks *et al*., 2014). The application of *Trichoderma* spp. has been associated with decreased *R. solani* diseases on crops as Jerusalem artichoke and potato (Ezzat *et al*., 2015; Hicks *et al*., 2014). Several reports attributed different *Trichoderma* effects in inhibiting plant pathogens in the soil through their high antagonistic and mycoparasitic activity (Bhattacharjee and Dey, 2014), along with direct effects on plants roots, increasing nutrients uptake, improving seed germination, and stimulation of plant defenses against biotic and abiotic stresses (Hicks *et al*., 2014; Bhattacharjee and Dey, 2014).

Biological control ability may be brought about the use of mixtures of biocontrol agents (Hicks *et al.*, 2014; Ezzat *et al.*, 2015). In this respect, some reports indicated that a combination of antagonistic bacteria with antagonistic fungi especially *Trichoderma* sp. showed increased plant protection than when being used individually. For example, Brewer and Larkin (2005) reported that combination of

Bacillus subtilis and *Trichoderma virens* improved resistance against *R. solani* on potato than these biocontrol agents being used individually. Ezziyyani *et al.* (2007) reported that the application of mixture of *T. harzianum* and *Streptomyces rochei* was more effective in controlling *Phytophthora* root rot in bell pepper. Similar results were obtained by mixing two compatible biocontrol agents, as *Bacillus subtilis* CA32 and *T. harzianum* RU01, to control the damping-off in *Solanum melongena* and *Capsicum annuum* caused by *Rhizoctonia solani* (Abeysinghe, 2009).

The main objective of this study was to evaluate the efficacy of *B. subtilis* and two isolates of *Trichoderma* spp. individually and in combinations with respect to the mode of application of biocontrol agents for protection of potato plants against *R. solani* under laboratory and greenhouse conditions.

MATERIALS AND METHODS

Source of organisms and microbial inoculation

B. subtilis ATCC®11774™ was obtained from American Type Culture Collection, (Illinois, USA). The stock culture of the bacterial strain was stored in 30% glycerol at -7 °C. Prior to each experiment, it was subcultured from the frozen stocks onto nutrient agar medium. The obtained bacterial cells were re-suspended in sterile 0.85% NaCl and centrifuged at 5000 rpm for 25 min at 4 °C. The supernatant was discarded and the washed bacterial cells were re-suspended in sterile distilled water. The concentration in the suspension was adjusted to 10^9 cfu ml^{-1}.

R. solani (AG3), isolated from infected potato tubers, was kindly supplied by the Vegetable Diseases Research Department, Plant Pathology Research Institute, ARC, Giza, Egypt. Inoculum was prepared by culturing on PDA plate and incubation at 25 ± 2°C for 3 days. Mycelium plugs were transferred to sterilized medium of sorghum: coarse sand: water (2:1:2, v/v/v) and incubated at room temperature for 10 days.

Two isolates *T. koningii* (Tk2) and *T. harzianum* (Th1) were previously isolated from the rhizosheric soil of health Jerusalem artichoke plants located at Baramoon Horticulture Research Station, Dakahlia Governorate, Egypt (Ezzat *et al.*, 2015). Each *Trichoderma* isolate was grown in a bottle containing sterilized sorghum : coarse sand : water containing (2:1:2 v/v/v) medium and incubated at 25±2ºC for 10 days, then the two inocula were mixed in equal portions. The identification of *Trichoderma* species was confirmed at the Mycological Center, Assiut University (AUMC), Egypt. Potato tubers (40-50 mm in diam.) of cultivar Valora were used in this study.

Dual culture assay:

In vitro antagonistic assay was performed according to the dual culture method on potato dextrose agar (PDA) medium (Difco, USA). A 5 mm disk of three-day old culture of *R. solani* was centrally placed in 9 cm Petri dish and *B. subtilis* was streaked in a square form around the agar disk at 2 cm distance. The antagonistic activity of *B. subtilis* was estimated by the inhibition of the fungal growth in comparison to a solely cultivated fungus. The decrease in fungal growth was monitored by measuring the diameter in centimeter of the colony until 5 days at 25+2 °C and 16 h light period (Elkahoui *et al.*, 2012).

The antagonistic potential of *T. hazianum* and *T. koningii* was evaluated against *R. solani* using dual culture technique (Dhingra and Sinclair, 1995). Five-millimeter mycelial disc of each test antagonist fungus taken from 5-day old culture was paired against the same sized mycelial disc of *R. solani* at the opposite end on 9 cm diameter PDA Petri plates. The pathogen and antagonist discs were placed at equal distances from the periphery of the Petri plate. The PDA plates inoculated only with the phytopathogen served as control. The plates were incubated at 25±2ºC. The growth of the pathogen in both tests and the control was recorded. The percent inhibition of radial growth = (R1–R2)/R1 × 100. Where R1 = radial growth of the pathogen in control. R2 = radial growth of the pathogen in dual culture with antagonists.

Greenhouse experiments:

The experiments were conducted at Tag Elazz Research Station, Dakahlia governorate, Egypt, during the summer seasons of 2012/13 and 2014/15. Plastic bags (50 cm in diameter) were filled with sterilized, clay: sand mixture (2:1, v/v). Healthy potato tubers were surface sterilized in 1% sodium hypochlorite, and then washed several times with sterilized water. The bags were singly infested with the previously prepared pathogen inoculum at the rate of 0.4% (w/w)., regularly watered, and left for one week to ensure even distribution of the pathogen. *B. subtilis* was applied at planting by adding 200 ml/bag of the bacterial suspension (10^9 cfu mL^{-1}) as soil drench. One week after inoculation with *R. solani,* two tubers (40-50 mm in diam.) were planted in each bag. Accordingly, 8 treatments were used; (1) Healthy control (uninoculated), (2) Diseased control (soil infested with *R. solani*), (3) Bacterized tubers, (4) *Tichoderma* spores mixture added tubers, (5) *B. subtilis* added to soil, (6) Trichoderma mixture added to soil, (7) *B. subtilis* added to tubers and *Trichoderma* mixture added to soil and (8) *Trichoderma* spores mixture added tubers and *B. subtilis* added to soil. Bags that were inoculated with *Trichoderma* mixture received 40 g of the inoculum/bag, as seed-bed before planting. In case of tubers treated by

Trichoderma mixture (10^8 spore mL^{-1}) and *B. subtilis* (10^9 cfu mL^{-1}), tubers were soaked in either fungal or bacterial suspensions for 20 min, then dried before planting. Another set of bags of disinfected soil and untreated tubers was used as a control. The experiment was arranged in a completely randomized block design. Nine replicates were used per treatment. Plants were grown in a greenhouse for 100 days at temperature ranging between 20 and 30 °C, soil pH was 7.9.

After six weeks from planting, five plants from each treatment were taken off, washed to evaluate the severity of stem canker developed based on a scale of (0-5) described by Brewer and Larkin (2005) as follow: 0 = no disease symptoms; 1= brown discoloration of stem; 2 = cankers covering < 25% of the stem circumference; 3 = 25-75% covered by cankers; 4 = 75% coverage by stem cankers; and 5 = completely nipped off or death of the plants. After harvest, newly formed sclerotia were visible on the tubers; therefore, black scurf was assessed on a scale of 0–5 according to Brewer and Larkin (2005). After 40 days from sowing, the total phenol content in potato plant was determined according to Malik and Singh (1980), as well as assay of polyphenol oxidase (Maxwell and Bateman, 1967) and peroxidase (Galeazzi *et al.*, 1981). After 70 days of sowing, photosynthetic pigments (chlorophyll and carotenoids) were measured according to Mackinney (1941). The growth was evaluated in terms of height and fresh and dry weight of shoot, the number of leaves and stolons. At harvest, number, fresh and dry weight of tubers were recorded. Starch contents were estimated using the equation of Burton (1948) as follows: Starch (%) = 17.546 + 0.89 (tuber dry matter % - 24.18)

Statistical analysis

The greenhouse experiment was arranged in one-way randomized blocks design, means comparison was performed based on least significant differences test. The statistical analysis; CoStat (CoHort Software, U.S.A) version 6.4 was used. The probability (*P*) value of ≤ 0.05 was used to evaluate the various treatments.

RESULTS AND DISCUSSION

Antagonism of the bioagents to *R. solani*

Antagonistic properties of both *T. koningii* (possible pathogen on sweet potato) and *T. harzianum* were tested through dual Petri plate method. Both *Trichoderma* species caused a pronounced decrease of the mycelial growth of *R. solani* reaching 70.0 and 74.29%, respectively, after 5 days of incubation (Table 1). However, eight days later *R. solani* was completely overgrown by *Trichoderma* mycelia of both species. Furthermore, sclerotia formation was lacking compared to the

control plates. This finding complies with many reports that asserted that *T. harzianum*, *T. virens* and *T. hamatum* are very effective at inhibiting mycelial growth of soil-borne, seed borne, phyllosphere and storage plant pathogens on PDA (Vinale *et al.*, 2014; Bhattacharjee and Dey, 2014). *Trichoderma* strains were reported to secrete many cell wall degrading enzymes during the mycoparasitic interaction with its hosts. In this respect, Chitinases and β -1,3-glucanases have been found to be directly involved, which allow them to bore holes into its host fungi and extract nutrients for their own growth. In addition, it strongly inhibited sclerotia production and suppressed sclerotia germination of pathogen (Naeimi *et al.*, 2010). Biocontrol isolates belonging to *Trichoderma* are well-known producers of different bioproducts that are toxic to phytopathogenic fungi. Among these metabolic byproducts, pyrones, koninginins, viridian, gliovirin, gliotoxin, peptaibols and others have been described (Vinale *et al.*, 2014). This may explain the obtained growth reduction of *Rizoctonia*.

Dual culturing of *R. solani* and *B. subtilis* was made. The assay showed marked retardation of pathogen growth. The dual culture should reduced growth of *R. solani* by 40.48% after 5 days (Table 1). No sclerotia were developed and cytoplasm of the mycelium showed dramatic changes when observed under microscope (data not shown). Mycelium closest to bacteria become yellow indicating some diffusates from bacteria had reached this part of the mycelium. According to Elkahoui *et al.*, (2012), *B. subtilis* might also act on pathogenic fungi by either producing antifungal substances or colonizing microsites faster than the surface fungi. This result corroborates with other works, which that *B. subtilis* produces reported lipopeptides belonging to the iturin and surfactin in the late phase of growth that inhibit *R. solani* growth (Elkahoui *et al.*, 2012).

Table 1. Inhibition of *R. solani* by *Trichoderma* species and *B. subtilis* ATCC 11774 by dual assay method.

Treatment	Decrease % (5 days)
R. solani + *T. koningii*	70.0 a
R. solani + *T. hazianum*	74.29 a
R. solani + *B. Subtilis*	40.48 b

Same letter(s) within a column, indicate non-significant difference ($P \leq 0.05$)

Interaction between *B. subtilis* (ATCC 11774) and *T. koningii* and *T. hazianum* fungi*:*

The antagonism test between *T. koningii*, *T. hazianum* and *B. subtilis* ATCC 11774 was carried out to ensure the compatibility of these species when used as combined inoculum. In this respect, *B. subtilis* (ATCC 11774) did not show any

antagonism against both *Trichoderma* species. Also, the test showed no visible antagonism between *T. koningii* and *T. hazianum*, which encourages the use of a mixture of the two *Trichoderma* species, as well as with *B. subtilis*.

Greenhouse bioagents evaluation :

All the biological control treatments showed variation in plant disease index compared to inoculated control (Table 2). The disease index was decreased significantly in all case compared to untreated-infected control. Bacterial tuber treatment alone significantly decreased the disease index of potato in both experiments being 79.88 and 72.75%. When *Trichoderma* was applied alone to tubers prior to sowing, the lowest protection levels observed, reached 60.1% and 54.50% in the first and second experiments, respectively. Tubers treatment with bacteria and soil application of *Trichoderma* showed a highly significant effect on protection of potato plant, being similar to that reached with soil application with *Trichoderma* mixture to be 89.49, 91.83% and 87.99, 91% in first and second experiments, respectively. Ciampi *et al.* (2007) found that using *B. subtilis* alone, led to 58.12% of healthy tubers compared with 24% in control treatments. Recently, chitinase production by *Bacillus subtilis* (ATCC 11774) and its effect as biocontrol agent of *Rhizoctonia* disease on potato was studied. Under greenhouse conditions, application of a bacterial suspension of *B. subtilis* significantly reduced the incidence and severity of stem canker (77.79 and 66.7%, respectively) and black scurf diseases (52.33 and 70.59%, respectively) compared to the infested control. In addition, it significantly improved biochemical parameters, growth and tubers yield (Saber *et al.*, 2015).

Table 2. Influence of *B. subtilis* ATCC 11774 and *Trichoderma* mixture (*T. koningii* + *T. harzianum*) on the development of stem canker of potato under greenhouse conditions

Treatment	Disease index			
	1st experiment	Decrease (%)	2nd experiment	Decrease (%)
Control (without any treatment)	0.0 d	-	0.0 d	-
Control (soil infested with *R. solani*)	3.33 a	-	3.67 a	-
Tuber treated with *B. subtilis*	0.67 b-d	79.88	1.0 c	72.75
Tuber treated with *Trichoderma* mixture	1.33 b	60.1	1.67 b	54.5
Soil infested with *B. subtilis*	1.0 bc	69.97	1.33 bc	63.76
Soil infested with *Trichoderma* mixture	0.4 cd	87.99	0.33 d	91
Tuber treated by *B. subtilis* and soil infested with *Trichoderma* mixture	0.35 cd	89.49	0.3 d	91.83
Tuber treated by *Trichoderma* mixture and soil infested with *B. subtilis*	1.33 b	60.1	1.0 c	72.75

Same letter(s) within a column, indicate non-significant difference ($P \leq 0.05$)

Growth characteristics as affected by the bioagents:

The data (Table 3) indicated that significant variation was recorded for growth parameters of potato plants. However, a significant increment in first and second experiments was observed in plant height due to combined tuber applications of bacteria with soil application of *Trichoderma* mixture (40.33 and 37.0 cm, respectively). Tuber or soil applications with *Trichoderma* mixture alone showed the maximum significant increase in number of branches in both experiments (3 and 3.33, respectively) as compared to infected control. Among all treatments, combined tuber treatments of *Trichoderma* mixture with soil applications of bacteria exhibited the highest increase of plant stolons and leaves numbers in the first and second experiments (4.67, 5.0 and 17.33, 18.33, respectively) as compared with infested control (1.67, 2.0 and 12.0, 10.33, respectively).

Table 3. Potato growth characteristics as affected by application with *B. subtilis* ATCC 11774 and *Trichoderma* mixture under greenhouse conditions

Treatment	Plant height (cm)		No. of branches plant-1		No. of stolons tuber-1		No. of leaves Plant-1		Fresh weight g-1		Dry weight plant g-1	
	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment
Control (without any treatment)	34.0 bc	31.0 b	2.0 ab	1.67 b	2.33 bc	2.0 bc	16.33 ab	15.67 a	45.63 bc	42.63 c	8.35 cd	7.61 c
Control (infected)	23.0 ef	20.0 e	1.33 b	1.67 b	1.67 bc	2.0 bc	12.0 d	10.33 e	32.1 d	29.1 e	7.00 cd	6.66 c
Tuber treatment with *B. subtilis*	19.33 f	16.33 f	2.0 ab	2.33 ab	2.0 bc	2.33 bc	14.0 cd	12.67 d	33.03 d	30.03 e	6.34 d	5.14 d
Tuber treatment with *Trichoderma* mixture	26.33 de	23.33 d	3.0 a	3.33 a	0.67 c	1.33 c	12.67 d	14.67 bc	49.32 ab	46.32 b	12.12 ab	11.12a
Soil infested with *B. subtilis*	28.67 d	25.0 cd	2.0 ab	2.33 ab	3.0 ab	3.0 b	14.0 cd	13.0 cd	41.62 c	38.62 d	9.85 bc	9.18 b
Soil infested with *Trichoderma* mixture	30.0 cd	27.0 c	3.0 a	3.33 a	2.33 bc	2.67 b	15.33 a-c	16.0 b	56.58 a	53.58 a	12.23 ab	10.90 a
Tuber treated by *B. subtilis* and soil infested with *Trichoderma* mixture	40.33 a	37.0 a	2.67 ab	2.33 ab	2.0 bc	2.33 bc	15.0 bc	16.33 b	53.97 a	50.87 a	13.13 a	11.63 a
Tuber treated by *Trichoderma* mixture and soil infested with *B. subtilis*	35.33 b	30.0 b	2.0 ab	2.67 ab	4.67 a	5.0 a	17.33 a	18.33 a	54.22 a	50.88 a	13.05 a	12.05 a

Same letter(s) within a column, indicate non-significant difference ($P \leq 0.05$)

On the other hand, no significant differences was observed in shoot fresh and dry weights in the two treatments. *Trichoderma* spp. are known as plant growth promoting fungi, they are able to produce various bioactive secondary metabolites, which stimulate plant growth and protect it against various phytopathogens (Vinale *et*

al., 2014). The ability to stimulate plant development is mediated by the activation of auxin-dependent mechanism and/or producing auxin analogues (Hicks *et al.*, 2014). Several secondary metabolites produced by *Trichoderma* spp. such as koninginins, 6PP, trichocaranes A-D, harzianopyridone, cyclonerodiol, harzianolide and harzianic acid affect plant growth in a concentration dependent manner (Vinale *et al.*, 2014). These reported results are in accordance with that obtained by Hicks *et al.* (2014) who reported the greatest potential of *T. harzianum* LU1491, *T. barbatum* LU1489 and *Trichoderma* sp. 792 LU1483 to promote potato plant growth parameters (number of tubers, total tuber weight, and average tuber weight, respectively) compared with the infected control by *R. solani*.

Effect of treatment with the bioagents on productivity and yield

Results of *Trichoderma* mixture as tuber treatment and bacteria added to soil significantly increased the number of tubers and total tuber weight by 33.25, 22.33% and 137.34 and 159%, respectively, in the first and second experiments. A significant increment was also recorded by the above-applied combination treatment in both tubers dry weight and starch contents (22.21, 20.71% and 29.24, 30.54%, respectively) . However, no significant differences were recorded among all biocontrol treatments in increasing mean tuber weight during both experiments. In this regard, Hicks *et al.* (2014) evaluated six *Trichoderma* strains under greenhouse and field conditions for stem canker suppression and growth promotion of potato plants.

Table 4. Effect of treatment with *Bacillus subtilis* and *Trichoderma* mixture on productivity and yield of potato

Treatment	No. of tubers Plant^{-1}		Weight of tuber (g)		Mean tuber weight (g)		Tuber dray weight (g)		Starch content (%)	
	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment
Control (without any treatment)	2.33 b	233 ab	137.13 d	127.13 d	60.84 a	56.60 ab	19.33 a	19.43 a	13.23 a	13.32 a
Control (infected)	2.67 b	2.33 ab	73.83 g	63.83 g	28.61 a	28. 47 b	15.85 d	16.03 e	10.26 d	10.15 e
Tuber treatment with *B. subtilis*	1.67 b	1.67 b	110.5 f	100.83 f	73.98 a	67.1 ab	19.00 bc	18.89 d	12.94 b	12.84 d
Tuber treatment with *Trichoderma* mixture	2.0 b	2.33 ab	135.18 de	125.17 de	67.59 a	55.53 ab	18.80 c	18.85 d	12.76 c	12.80 d
Soil infested with *B. subtilis*	2.33 b	2.0 ab	132.59 e	123.28 e	73.98 a	75.23 a	19.03 b	19.13 bc	12.97 b	13.06 c
Soil infested with *Trichoderma* mixture	2.33 ab	2.67 ab	156.27 c	146.6 c	69.42 a	56.87 ab	18.90 bc	19.00 cd	12.85 bc	12.94 cd
Tuber treated by *B. subtilis* and soil infested with *Trichoderma* mixture	2.33 b	2.33 ab	171.1 b	161.67 b	67.14 a	71.94 a	19.25 a	19.18 b	13.16 a	13.10 bc
Tuber treated by *Trichoderma* mixture and soil infested with *B. subtilis*	4.0 a	3.0 a	175.23 a	165.33 a	43.81 a	55.11 ab	19.37 a	19.35 a	13.26 a	13.25 ab

Same letter(s) within a column, indicate non-significant difference ($P \leq 0.05$)

Generally, combination treatments provided the greatest disease suppression as well as increasing plant growth parameters. In this respect, *T. atroviride* LU144 alone and in combination with other *Trichoderma* strains gave the highest significant effect on potato shoot nipping, number of stolons, number of symptomless stolon tips and the number of tubers. The yield increase determined in the present study may have resulted either from suppression of *Rhizoctonia* infection or from direct interactions between the *Trichoderma* strains and the potato plants, in terms of hormonal regulation or nutrient acquisition (Bhattacharjee and Dey, 2014); or possibly a combination of these effects.

Physiological activities of plants

As shown in Table (5), Chl a, Chl b and total Chls significantly increased in potato plants resulting from tuber treatment with *Trichoderma* mixture and bacteria added to soil. Soil application alone with *Trichoderm*a mixture came next in this respect. On the other hand, no significant differences were recorded among all treatments and infected control for carotenoids content. The increment in chlorophyll content, which is a good parameter reflecting the health condition of plant, enhancing the efficacy of photosynthetic apparatus with a better potential for disease resistance (Amaresh and Bhatt, 1998).

Table 5. Effect of treatment with *Bacillus subtilis* and *Trichoderma* mixture on chlorophyll contents of potato plant

Treatment	Chlorophyll contents (mg/g Fresh weight)							
	Chla		Chl b		Total chls		Carotenioid	
	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment
Control (without any treatment)	1.157 bc	1.316 bc	1.108 b	1.266 b	2.265 b	2.581 b	0.057 b	0.271 ab
Control (infected)	1.028 c	1.282 bc	0.496 d	0.565 e	1.524 d	1.847 c	0.174 ab	0.307 a
Tuber treatment with *B. subtilis*	0.813 d	0.927 cd	0.712 cd	0.803 d	1.525 d	1.730 c	0.080 ab	0.152 ab
Tuber treatment with *Trichoderma* mixture	1.198 b	1.821 a	1.104 b	1.361 ab	2.303 b	3.182 a	0.083 ab	0.198 ab
Soil infested with *B. subtilis*	1.244 b	1.553 ab	0.785 c	0.898 cd	2.030 c	2.450 b	0.124 ab	0.264 a
Soil infested with *Trichoderma* mixture	1.250 b	1.455 ab	0.895 bc	0.998 c	2.144 bc	2.454 b	0.100 ab	0.264 ab
Tuber treated by *B. subtilis* and soil infested with *Trichoderma* mixture	0.636 e	0.688 d	0.135 e	0.345 f	0.770 e	1.033 d	0.208 a	0.295 a
Tuber treated by *Trichoderma* mixture and soil infested with *B. subtilis*	1.415 a	1.770 a	1.391 a	1.523 a	2.806 a	3.293 a	0.043 b	0.119 b

Same letter(s) within a column, indicate non-significant difference ($P \leq 0.05$)

The acquisition of carbon is strongly modulated by the surface area of photosynethesizing leaves; hence, leaf area development is germane to the efforts to increase yield (Kays and Nottingham, 2008). Total phenols, polyphenoloxidase and peroxidase activity in potato plants were determined (Table 6) since they play important role in plant protection.

Table 6. Biochemical activities of potato as affected by the inoculation by *Bacillus subtilis* and *Trichoderma* mixture under greenhouse conditions

Treatment	Peroxidase (Unit^{-1} min g^{-1} fresh wt.)		Polyphenoloxidase (Unit^{-1} min g^{-1} fresh wt.)		Total phenol (mg catechol 100 g^{-1} fresh wt.)	
	1st experiment	2nd experiment	1st experiment	2nd experiment	1st experiment	2nd experiment
Control (without any treatment)	0.713 b	0.7718 b	1.017 b	1.510 a	39.87 bc	42.0 c
Control (infected)	0.333 d	0.3548 d	0.757 b	0.822 bc	28.52 c	30.67 c
Tuber treatment with *B. subtilis*	0.567 c	0.5708 c	0.787 b	0.729 c	29.85 c	33.0 c
Tuber treatment with *Trichoderma* mixture	0.573 c	0.6333 c	0.870 b	0.918 bc	32.52 bc	35.67 c
Soil infested with *B. subtilis*	0.730 b	0.7478 b	0.990 b	1.005 bc	30.52 c	35.0 c
Soil infested with *Trichoderma* mixture	0.313 d	0.4060 d	1.378 a	1.300 ab	161.42 a	158.0 a
Tuber treated by *B. subtilis* and soil infested with *Trichoderma* mixture	0.930 a	0.9019 a	0.7208 b	0.766 c	45.21 b	38.0 c
Tuber treated by *Trichoderma* mixture and soil infested with *B. subtilis*	0.843 a	0.8705 a	1.387a	1.511 a	153.40 a	132.33 b

Same letter(s) within a column, indicate non-significant difference ($P \leq 0.05$)

Results showed a significant increase in peroxidase activity due to both dual application treatments in both experiments. On the other hand, combined tuber treatment with *Trichoderma* mixture and soil application of bacteria, were similar to that reached with soil application with *Trichoderma* mixture that showed significant increase in polyphenoloxidase and total phenol contents as compared to infested control. Varied mechanisms have been discussed in the context of direct and/or indirect microbial antagonism. The predominance of one of these mechanisms does not exclude the contribution of one or more of the other mechanisms (Bhattacharjee and Dey, 2014). Direct mechanisms include microbial competition for space and/or nutrients, antibiosis *i.e.* production of some antagonistic metabolite such as antibiotics, antifungal enzymes, toxic volatile and non-volatile compounds, and mycoparasitism (deriving nutrients from the pathogenic fungus). While, the indirect mechanisms include defense responses induction in the host plant or plant growth enhancement resulting in more vigorous and resistant plants (Vinale *et al.*, 2014).

Recent reports suggested that *Trichoderma* isolates might stimulate the production of biochemical compounds of phenolic nature associated with the host defense (Surekha *et al.*, 2014). The activity of defense-related enzymes can substantiate the host resistance against plant pathogens. The increase in activity and accumulation of these enzymes also depend on the plant genotype, physiological conditions and the type of pathogen. Synthesis of defense chemicals against pathogens is triggered by a series of morphological and biochemical changes initiated by specific strains of fungi (Surekha *et al.*, 2014). Accumulation of phenols might be due to excess production of H_2O_2 in infected plants through increased respiration or due to the activation hexose-monophosphate shunt pathway, acetate pathway and release of bound phenols by hydrolytic enzymes (Goodman *et al.*, 1967). These biochemical reactions might have mediated antimicrobial activity followed by increased esterification of phenylpropanoids of the cell wall (Mandal and Mitra, 2007). Similarly, synthesis of high amount of the resistance enzymes and phenols by *Trichoderma* mixture and its combinations with bacteria, compared to infected controls, suggests their role in inducing resistance against stem cankers and black scurf in potato.

In the present investigation, the use of *T. koningii* and *T. harzianum* (in mixture) as well as combined with *B. subtilis* showed high efficacy for suppression of *Rhizoctonia* disease as well as promotion growth and productivity of potato. This encourages the incorporation of such compatible bioagents for the *Rhizotonia* disease management and the production of potato. The success of such bioagents under greenhouse conditions promotes further research under field conditions with such treatments.

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نوعان من *Trichoderma* وبكتريا *Bacillus subtilis* كعوامل مقاومة حيوية لأمراض الريزكتونيا والتأثير على زيادة الإنتاج في البطاطس

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تعد أمراض تقرح السيقان والقشرة السوداء المتسبب عنه فطر *Rhizoctonia solani* أحد المشاكل الهامة الى تواجه زراعة البطاطس. وهدفت هذه الدراسة التي تمت تحت ظروف الصوبة، الى تقيم استخدام ثلاثة عوامل حيوية متجانسة وتشمل *Trichoderma koningii* و *T. harzianum* (مخلوطان) و *Bacillus subtilis* ATCC®11774™ في صورة فردية أو مخلوطة مع كلا الفطرين تحت ظروف الصوبة في تثبيط المرض وزيادة النمو في نباتات البطاطس. حيث أظهرت كل العزلات كفاءة تضادية مع فطر *Rhizoctonia solani* على أطباق بتري تحت ظروف المعمل. وتحت ظروف الصوبة أظهرت معاملتي إضافة البكتريا للدرنات أو اضافة خليط التريكوديرما للتربة، خلال تجربتين، قدرتهما على خفض تواجد المرض في نباتات البطاطس. كما وجد أن إضافة خليط التريكوديرما للتربة بصورة منفردة أو بالجمع مع معاملة الدرنات بالبكتريا أظهر أعلى تأثير مثبط لأعراض التقرح في نباتات البطاطس. وبالنظر الى تشجيع النمو، أظهرت معامله الجمع بين إضافة البكتريا للدرنات واضافة خليط التريكوديرما للتربة الأفضلية في زيادة طول النباتات. وأظهر التلقيح المزدوج بين اضافة خليط التريكوديرما للدرنات مع إضافة البكتريا للتربة الأفضلية في زيادة أعداد السيقان والأوراق في كلا التجربتين. كما أظهرت معاملتي التلقيح المزدوج وكذا اضافة خليط التريكوديرما للتربة بصورة منفردة تأثير معنوي كبير في زيادة الوزن الطازج والوزن الجاف لنباتات البطاطس. وكذا زيادة معنوية في كل من انتاج الدرنات وبعض الصفات البيوكيميائية (محتوى الكلوروفيل والفينولات الكلية وانزيمات البيروكسيديز والبولى فينول اوكسيديز). وتقترح هذه النتائج إدراج تلك العوامل الحيوية في مقاومة أمراض *Rhizoctonia* وزيادة انتاجية البطاطس.