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CONTROLLING STEM CANKER AND BLACK SCURF DISEASE COMPLEX ON POTATO PLANTS USING *TRICHODERMA* ISOLATES

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A B S T R A C T

Rhizoctonia solani, the causal agent of stem canker and black scurf disease on potato is a real threat to potato crop with up to 50% yield reduction. Current study was carried out to determine the effect of some *Trichoderma* isolates with different types of soil on the above diseases under laboratory and glasshouse conditions. Eight non-pathogenic strains of *Trichoderma* were isolated from potato roots and surrounding soil and examined for antagonism of Rhizoctonia diseases. Results showed that strains *T. hamatum* T8 and *T. harzianum* T5 had great potential as biocontrol agents for inhibiting the pathogen growth in dual culture and culture filtrate methods. The effect of different types of soils on the radial growth of *R. solani* AG-3PT in plates showed that Dorrigo soil inhibited the pathogen growth in laboratory by 68% as the colony diameter of pathogen recorded 2.8 cm which exceeds other soil types. While most *Trichoderma* strains that isolated from this area had a potential of protecting potatoes from the infection by the pathogen and acted as a biocontrol agent. Applying non-pathogenic *Trichoderma* isolates was the best method when inoculated the soil of pots with conidial suspensions which promote the growth of potato plants. *T. harzianum* (T5), *T. hamatum* (T6) and *T. hamatum* (T8) strains were eliminated stem canker and black scurf disease and improved potato plant growth in glasshouse experiment. The outcome of current study indicated the potential for utilizing Australian *Trichoderma* strains to reduce Rhizoctonia diseases on potato plants and increase productivity.

Keywords: *Rhizoctonia solani* AG-3PT, Sebago, Biocontrol agent, Growth promotion.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an essential vegetable crop and ranked as a fourth main food after rice, maize and wheat in terms of area cultivated, yield and consumption (Taylor 2013; Marfil*et al.,* 2015). Due to the high value of this crop, the production of potatoes has grown in many countries, particularly in Europe and Asia (Fiers*et al.,* 2012). Many studies reported that various microorganisms including fungi, bacteria, viruses and nematodes attack potato plants causing significant diseases and reducing its productivity (Demirci*et al.,* 2009; Fiers*et al.,* 2012; Alshimaysawe, 2018). The anastomosis group 3 of *Rhizoctonia solani* (AG-3PT) is an economically important fungus that causes stem canker as well as black scurf diseases on plants and potato tubers (Demirci*et al.,* 2009). The both diseases can cause 35-50% yield loss annually in the infected farms (Banville 1989).

R. solani has various strains and types spreading worldwide, and can be found in all kind of soils (Taylor 2013). *R. solani* affects potato plants at all stages of growth either underground or aboveground plant parts. Symptoms of disease including sprouts death, cankers on stolons and underground parts of stem, reduction of root system and sclerotia on tubers (Ikeda*et al.* 2012). Fungicides have been used to control plant pathogens as an effective strategy, however, it has short term control and many consequences such as harmful residues of these chemicals to human health and environment (Kumar*et al.,* 2017). As a result, many attempts have been conducted to find alternatives methods to replace fungicides (Al-

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Numerous studies showed that fungal biocontrols can be reduced or inhibited the severity and incidence of *R. solani* disease such as *Trichoderma* spp., *Bacillus subtilis* and *Verticillium biguttatum* (Mayo*et al.,* 2015). It is well known that *Trichoderma* spp can limit the growth of pathogens and enhance the growth of plant. *Trichoderma* species develop resistance in plant, promote its growth, compete pathogens for nutrients and space as well as it works as mycoparasitism and secret the antibiotics (Devi*et al.,* 2017). *Trichoderma* species could be stimulated potato plant growth and increased yield production by eliminating or inhibiting soilborne pathogen (*R. solani*) (Waghunde*et al.,* 2016). Therefore, current study was conducted to examine *Trichoderma* strains to eliminate AG-3PT group that causes diseases on potato, and also select the high effective biocontrol and strong promotion of growth to be used and deployed.

MATERIALS AND METHODS

Isolation, identification of *Trichoderma* isolates: *Trichoderma* species were obtained from potato roots (free of stem canker and black scurf symptoms) and surrounding soil that collected from potato farms in Dorrigo region, NSW, Australia in January 2015. Roots washed to remove the surrounding soil, and transferred to laboratory in plastic bags. Roots were sterilized by 1% NaOCl for 1 min, after that the roots were washed many times with sterilized distilled water (S.D.W.) to remove NaOCl residues. Roots were cut to 1 cm pieces and placed on *Trichoderma* selective media (T.S.M.). Plates were incubated at 25°C and the fungal growth checked daily for one week. Soil from potato field was diluted using distilled water then from the last dilution (10^{-4}) one ml was poured in fresh plates contained T.S.M. medium. After that, hyphal tips from the edge of each colony were taken and transferred onto fresh plates containing P.D.A. medium, then incubated at 25°C for one week. Eight *Trichoderma* isolates (T1, T2, T3, T4, T5, T6, T7 and T8) were isolated. Morphological characteristics were studied using microscope to identify *Trichoderma* isolates (Elad and Chet, 1983). Treatments 1, 2, 4 and 5 were identified as *Trichoderma harzianum*, while Treatments 3, 6 and 8 were identified as *Trichoderma hamatum*. T7 was identified as *Trichoderma* sp. The identification of *Trichoderma* isolates was confirmed by extracting DNA following Cenis (1992) procedure and obtaining partial sequences of ITS. Sequences were submitted to the GenBank (NCBI) through (https//submit.ncbi.nlm.nih.gov/ subs/ genbank/). GenBank accession numbers were MH675470 for T8 and MH675469 for T5.

Preparation of pathogenic fungal inoculum and testing the pathogenicity: AG-3PT isolates of *R. solani* were collected from Agricultural Scientific Collections Trust, NSW, Australia. The inoculum of pathogen was made by mixing 200 g of wheat seeds with 400 mL water in 1000 mL bottles and left overnight. Seeds of wheat were autoclaved for one hour in two days (1 h per day). Once bottles were cooled, 10 discs (0.5 cm diameter) form fungal colonies of the pathogenic fungus grown on P.D.A. for 6 days were taken to inoculate these bottles. The contents of bottle were shaken every two days (Baijnath 2012). Potato tubers of Sebago cultivar sterilized with 1% of sodium hypochlorite (NaOCl) for 2 min and rinsed by S.D.W. to test the pathogenicity of the fungal pathogen. Potato tubers were sown into plastic pots (20 cm diameter), filled with potting mix soil (non-sterile) mixed with washed river sand in 2:1 ratio (Alshimaysawe 2018). Five replicates for each treatment were used in this glasshouse study which continued for five weeks at 20°C . Indicators of *R. solani* pathogenicity were noted by the visible symptoms of the disease on tubers.

Dual plating assays: *R. solani* AG-3PT was cultured with eight isolates of *Trichoderma* at 22°C and incubated for five days in the same plates at 6 cm distance of on P.D.A. 5 mm diameter of mycelial plugs from five days old colonies of both the pathogen and the fungal biocontrol were used. The pathogenic fungus was planted near one of plate edges alone as a control. The percentage of inhibition % of the radial growth of *R. solani* AG-3PT was measured according to the equation:

$$Inhibition = \frac{\mathrm{R1} - \mathrm{R2}}{\mathrm{R1}} X100$$

R1 represent AG-3PT mycelial growth in control, R2 represent the grown distance of the pathogenic fungus on a line between the inoculated *R. solani* and the fungal biocontrol isolates (Subhani *et al.,* 2013; Aydin, 2018).

Antibiosis assays: Isolates of *Trichoderma* (the biocontrol agent) were cultured on 150 mL tubes containing 40 mL of sterilized medium of potato dextrose broth (P.D.B.) and then tubes incubated at 22°C on a shaker for 7 days at 140 rpm. *R. solani* pathogen was cultured in P.D.B. to be used as a control. The biomasses of fungi used were filtered through Whatman 41 filter paper, and then 0.45 µm membrane was used to sterilize them. 5 mL of the filtrated solution was poured in sterile fresh

plates contain 15 ml (2 g\L of glucose) of low sugar P.D.A. then it gently mixed. 5 mm diameter disc from five days old colony of the fungal pathogen was used to inoculate the center of the plates which then subjected to incubation for five days at 22°C. *R. solani* (AG-3PT) mycelial growth was measured then a comparison with the growth of control was done (Chen*et al.,* 2012).

Effects of different types of soil on the radial growth of pathogen *in vitro*: Different types of soil were tested to evaluate their effect on the radial growth of *R. solani* AG-3PT in Petri plates. Six types of soil were collected from different areas Trevenna Farm (Chromosol loamy sand, total 1.573% C, 0.161% N, 10.6% silt, 74.3% sand, 14.5% clay, exchangeable K 0.39 cmolc/kg, P 22.7mg/kg, pH 5.6 and EC 60.6 mS/cm), Laureldale Research Station (Vertosol heavy clay, parent material basalt, field capacity 23.8%, pH 5.98, ECE 26.71%, nitrate 31 µg /g), Smith-Road (pH 7.60, Ca 3.56, Fe 1.21, Mg 3.32, K 66.5, P 3.36), Dorrigo region (red soil or red Ferrosol which have high organic fertilizers, pH 5.7), Kirby soil (pH 5.50, EC 21.0, P 2.7 mg/kg) and 2:1 potting mix + sand, at 0-15 cm depth. The medium prepared from adding 100 mL of D. W. to 25 g of each soil after sieving (2 mm) in 250 mL flask, and then adding 2.25 g of agar to 25 mL of distilled water in 250 mL flask**.** Soil extract was added to the agar, and completed the total volume of suspension to 125 mL, and the autoclave used to sterile the medium for 25 min. At 45°C, and then poured into sterile plates. Three replicates of plates for each treatment were inoculated with 5 mm disc of the fungal pathogen at the center and then incubated for five days at 22°C, after that the mycelial growth of AG-3PT isolate was determined.

Effect of bio-control agent isolates on potato seedlings growth from tissue culture by root dipping and irrigation system: Seedlings of potato (cv. Sebago) were obtained from tissue culture which cultured for 6 weeks at the laboratory of tissue culture on fresh MS medium. Treatments 8 and 5 were cultured in plates contain P.D.A. medium, after that, plates were incubated at 25±2°C for five days. To evaluate the growth, a root dip inoculation technique was used. Seedling of Potato uprooted carefully and the roots rinsed under tap water to get rid of any dirt, sterilized by 1% of NaOCl for thirty seconds, rinsed under tap water for 3 more times. Roots were put in the conidial suspension (10^6 conidia/mL) of every isolate and then one seedling was shifted to 15 x 15 cm plastic pot filled with sandy-potting mix. However, seedlings that used as control treatment were soaked in S.D.W. only. Conidial suspension of every isolate was put to the same plastic pots of transplanted potato seedlings as a second evaluating method of the growth. to. S.D.W. was used to irrigate seedlings of control treatment only. Seedlings were put in greenhouse conditions at 20°C for three weeks (Rabeendran*et al.* 2000), and then the dry weight of root and shoot of seedlings was measured.

Effect of antagonists on the pathogenic fungus and potato growth: Experiments were conducted separately, used sandy-potting mix with vermiculite at 20°C in glasshouse for 7 weeks after emergence. In each experiment, there were ten treatments for each *Trichoderma* isolate with the fungal pathogen, a control treatment without inoculation, and one treatment for the pathogen alone. Seeds of Sebago cultivar were sterilized by NaOCl 1% for 2 minutes with 4 times of washing with S. D.W. Then planted in 16 cm diameter pots 16 g/pot of the inoculum of pathogen was added around the potato seeds. Seeds then soaked in 10^6 conidia/mL water suspensions of every *Trichoderma* isolate for one hour, dried for 3 h before sawing. Sclerotia number on potato tubers calculated eventually. Lesion length on stems was measured using 0-4 scale as follows:

Score	Symptoms of the disease
0	free of disease
1	stem covered with less than 10% of pathogen lesions
2	stem covered with 10-25% of pathogen lesions
3	stem covered with 26-50% of pathogen lesions
4	stem girdled with lesions

Potato growth was determined by calculating the dry weight of root and shoot, tubers and stolons number, and fresh weight of tuber. Oven was used at 60°C to dry shoots and roots until getting the dry weight (Atkinson 2005; Atkinson *et al.,* 2010; Alshimaysawe, 2018).

STATISTICAL ANALYSIS

All experiments were repeated once and data analyzed using Genstat (version 12 UK). To compare the means, the least significant difference (L.S.D.) was used at 5% level of significance (P>0.05).

RESULTS

Dual plating assays: There were significant effects of all tested *Trichoderma* strains which inhibited clearly the radial growth of *R. solani* AG-3PT pathogen on PDA with different degrees (Figure 1). The highest inhibition percentage of the radial growth of pathogen was noted in T8 (56.53%) followed by T2, T7 (53, 86 and 53.33%) respectively, whereas the lowest percentage of inhibition recorded in T5 (44.53%) followed by T4 (49.33%).

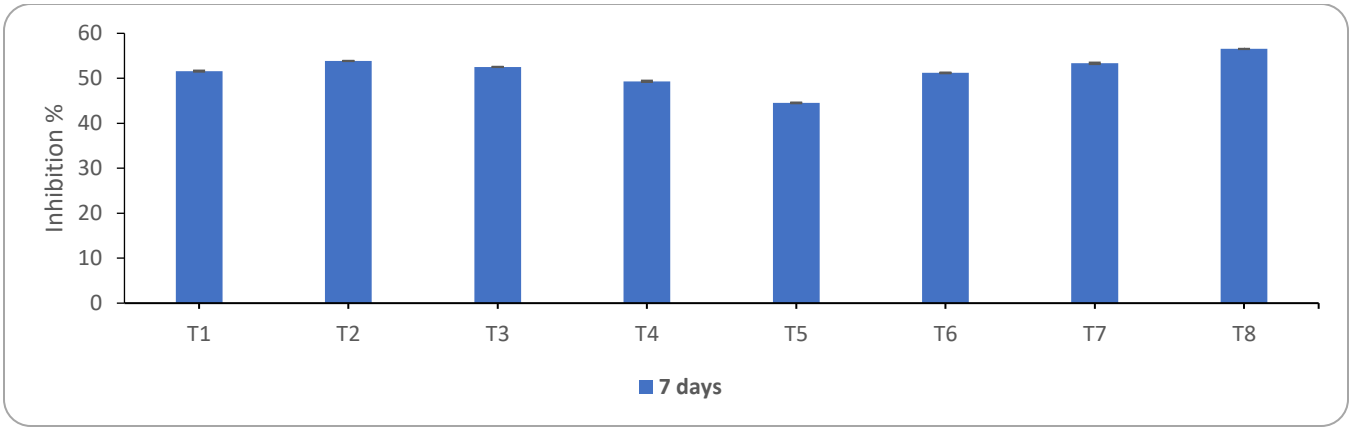

Figure 1. Inhibition the radial growth of *R. solani* by T1 to T8 isolates of *Trichoderma* using dual culture method on P.D.A. Standard errors was represented as error bars (n=3).

Antibiosis assays: *R. solani* radial growth was significantly inhibited by all *Trichoderma* isolates culture filtrate, except T6 that had no significant differences with the controls (Figure 2). While was strong inhibition of pathogen growth occurred by filtrates of T5 and T1 in compared to control treatments.

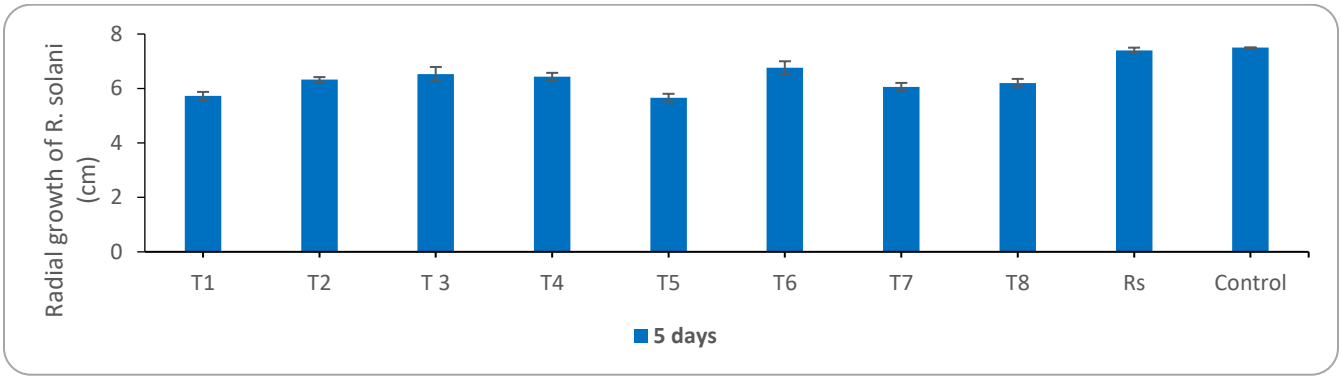

Figure 2. Effect of culture filtrate of T1 to T8 *Trichoderma* isolates on *R. solani* in plates of P.D.A., Standard errors was represented as error bars (n=3)

Effect of *Trichoderma* isolates on potato seedlings growth from tissue culture by root dipping and irrigation system: There was significant difference of adding T5 and T8 isolate conidial suspension and root dipping to potato plants with the absence of fungal pathogen. Growth promotion of *Trichoderma* isolates as a result of adding the suspension of conidia to the soil was stronger than root dipping technique (Figure 3 and Figure 4). Adding conidial suspension of *Trichoderma* through the root colonization significantly increased dry weight of shoot by 2.4 and 2.3 times for T8 and T5 respectively, dry weight of root by 2.3, and 2.2 times for T8 and T5 respectively, compared with untreated control (Figure 3 and Figure 4).

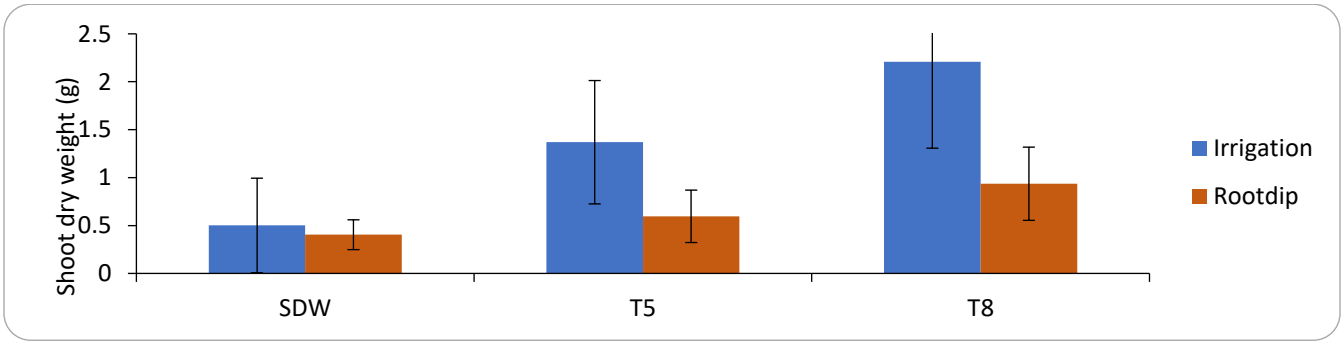

Figure 3. The effect of *Trichoderma* isolates (T5 and T8) on the dry weight of shoot of potato seedlings after inoculating the soil of pots with root dipping and the suspension of conidia. Standard errors were represented as error bars (n=3).

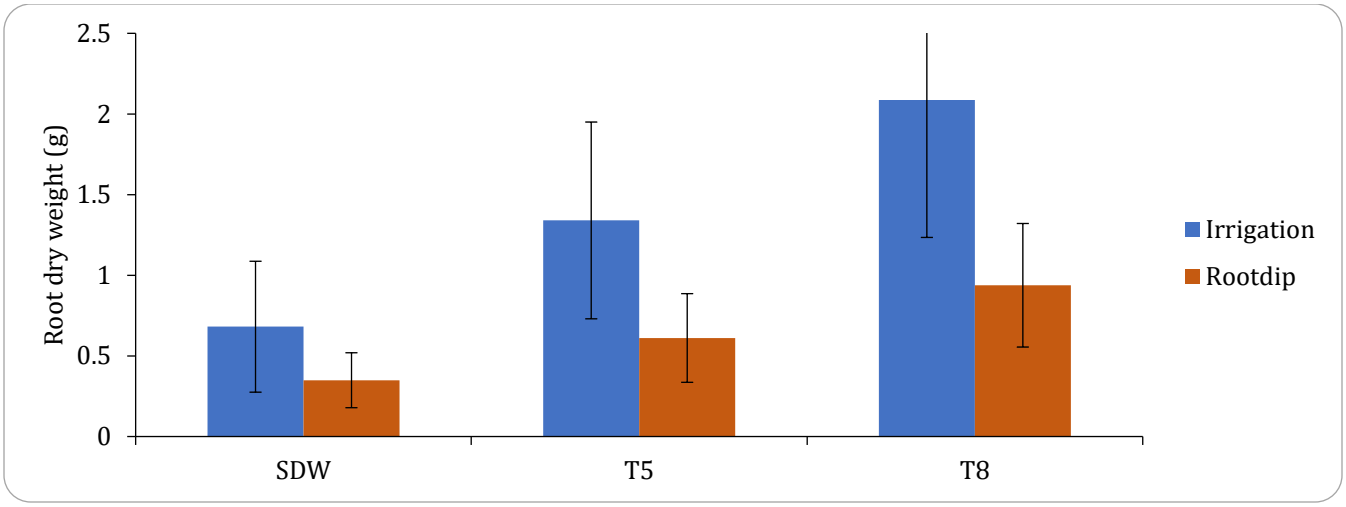

Figure 4. The effect *Trichoderma* isolates (T5 and T8) on the dry weight of root of potato seedlings after inoculating the soil of pots with root dipping and the suspension of conidia. Standard errors was represented as error bars (n=3).

The effect of soil type on the pathogen growth: The examination of different types of soils on *R. solani* AG-3PT mycelial growth in plates showed that, the growth of the pathogen was significantly lower in Dorrigo soil (Red soil) than the other treatments, whereas Trevenna soil (Loamy sand), Smith Road soil and sand with potting mix gave the highest growth rate of the pathogen (Figure 5).

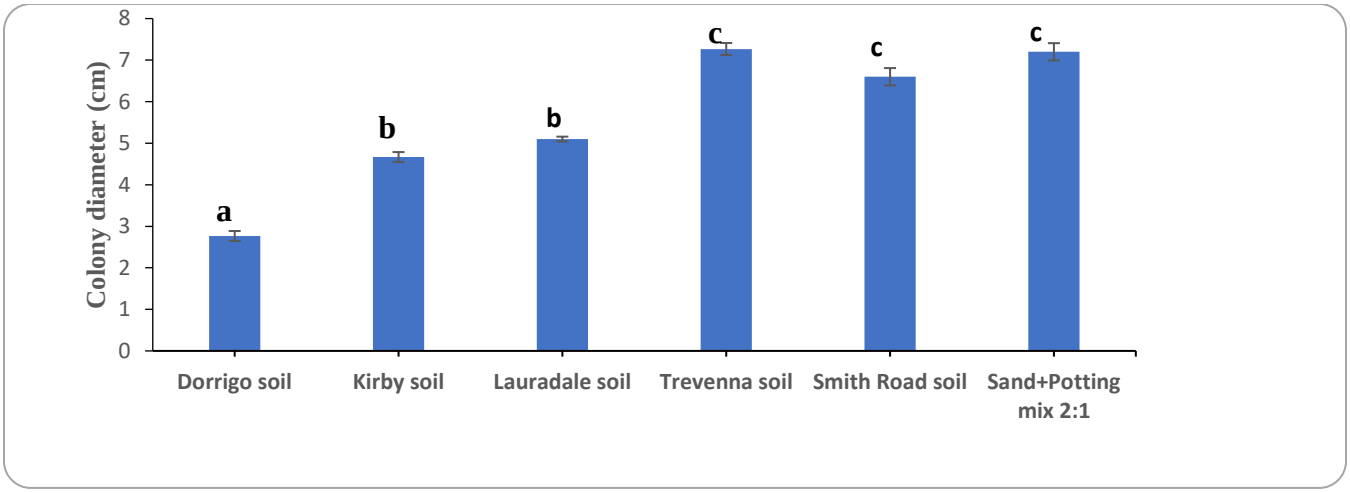

Figure 5. Colony diameter of *R. solani* on soil-agar-.medium after 5 days, Standard errors was represented as error bars (n=3), same letters on columns mean they are not differ significantly (P>0.05)

Effect of antagonists on stem- canker and black -scurf disease and the growth of potato: The outcomes indicated a significant effect of pathogen on the biomass production and productivity in vermiculite and the mix of sand potting in greenhouse conditions (Table 1 and 2). In vermiculite treatment, canker disease severity was 10-25% on stem and tubers showed a little number of sclerotia [9.69 plant/sclerotia (S. E. 1.072)] in *R. solani* AG-3PT trail only, and 0-10% on stems with brown lesions for T1, T2, T3, T4 and T7 plus *R. solani*AG-3PT. The interaction between T5 or T6 or T8 and pathogen treatment showed that the potatoes are free of the disease. There were no sclerotia on tubers in the non-inoculated control treatment. *R. solani* pathogen was reduced all parameters of growth as well as productivity of potato plants in comparison to other trails, but the differences of some other treatments and non-inoculated control were not significant, except the number of tubers. T5, T6 and T8 prevented the infection of plants by pathogen when treated plants with these isolates by showing free disease/sclerotia on tubers. Most *Trichoderma* isolates had significant greater dry weight of shoot, tubers number and fresh weight of tuber than the AG-3PT treatment only, particularly *T. hamatum* (T8) (Table 1).

Table 1. The effect of *Trichoderma* isolates (T1-T8) on reduction of stem canker and black scurf on Sebago cultivar planted in vermiculite under greenhouse conditions at 20°C

Treatment	Dry weight of shoot (g)	Dry weight of root (g)	No. of stolons	Tubers number	Tuber fresh weight (g)
T1 + Rs	1.05	0.32	1.67	2.00	35.89
T2 + Rs	0.90	0.26	1.33	2.67	44.33
T3 + Rs	1.06	0.19	1.50	2.50	21.08
T4 + Rs	1.07	0.12	1.67	1.67	15.81
T5 + Rs	0.99	0.21	1.50	1.50	20.17
T6 + Rs	1.14	0.26	2.33	2.67	37.14
T7 + Rs	1.03	0.28	2.00	2.00	33.87
T8 + Rs	1.21	0.29	1.67	2.33	48.72
Control	0.79	0.14	1.33	2.00	18.48
Rs	0.68	0.13	0.67	1.00	17.88
L.S.D	0.578	0.126	0.995	0.8	13.2

The least significant difference = P>0.05

In the second trial, stem canker disease was appeared in *R. solani* AG-3PT treatment at 10-25% of stem area infected, and 0-10% of stem covered with cankers for treatments of pathogen and T1 or T2 or T3 or T4 or T7. T5, T6 and T8 plus AG-3PT isolate showed free canker on the stems. Potato tubers inoculated with pathogen only showed 11.9 sclerotia/plant (S.E. 2.1); however, there were no sclerotia on tubers in un-inoculated control (plant only) or treatment of *Trichoderma* with pathogen.

AG-3PT treatment was decreased parameters of potato plant growth in comparison with control treatment (non-inoculated) in sand potting mix, but it was not significant (Table 2). All *Trichoderma* isolates were significantly augmented shoot dry weight, except T3 and T6 which were not significant compare to the treatment of pathogen only. T8, T1, T5, and T7 were increased the root dry weight significantly in comparison to AG-3PT only. Also, T8, T1, T2, T5, T6, and T7 were increased stolons number significantly, while results showed the number of tuber were not significantly differing in all treatments. All *Trichoderma* and pathogen treatments were promoted the tuber fresh weight significantly in comparison to AG-3PT only, except T4 which was not significant. Overall, T8 showed better effectiveness on the vegetative growth parameters of potato and protected plants from the disease.

Table 2. The effect of *Trichoderma* isolates (T1-T8) on reducing stem canker and black scurf on Sebago cultivar planted in mix of sandy potting under greenhouse conditions at 20°C

Treatment	Dry weight of shoot(g)	Dry weight of root (g)	No. of stolons	Tubers number	Tuber fresh weight (g)
T1 + Rs	2.67	0.96	3.33	2.33	29.74
T2 + Rs	1.92	0.85	4.33	3.33	31.65
T3 + Rs	1.24	0.79	2.67	2.67	29.72
T4 + Rs	1.69	0.75	2.50	2.5	21.69
T5 + Rs	2.81	0.97	3.50	3.33	38.55
T6 + Rs	1.37	0.86	3.00	3.00	34.48
T7 + Rs	3.23	0.94	4.00	3.00	31.24
T8 + Rs	3.39	1.08	5.33	3.67	44.92
Control	1.18	0.74	2.67	3.00	27.35
Rs	0.82	0.60	1.33	2.33	16.06
L.S.D	0.676	0.303	1.435	1.354	11.53

The least significant difference = P>0.05

DISCUSSION

In the present study, eight strains of *Trichoderma* including *T. harzianum*, were examined in vitro and glasshouse trials for the potential of using them as biocontrol agents against the pathogenic fungus (*R. solani* isolate: AG-3PT) the causal agent of potato stem -

canker. *T. hamatum* (T8) was reduced the radial growth of pathogen strongly in dual culture and augmented potato plant growth, whereas *T. harzianum* (T5) was more suppressive to AG-3PT pathogen in the antibiotic production method than others and also augmented growth of plants. Both isolates of *Trichoderma* (T8 and T5) have been produced highly toxic substances of the secondary metabolites as well as excreted enzymes that degrade cells wall which led to dissolve the wall of pathogen cells including chitinase and protease, competition for space, nutritional substances and production of appressoria to touch the pathogen and after that coiling around wall of mycelium of the fungus as well as parasitizing on the pathogen (Kredics*et al.,* 2003; Benitez*et al.,* 2004; Kumar*et al.,* 2016; Alshimaysawe, 2018). Many studies have been indicated that *Trichoderma* isolates encouraged potato vegetative growth through root colonization that could be led to change metabolism of plant, and also alters the phenolic compounds content, amino acids, hormones, photosynthesis rate (water content and transpiration) and soluble sugars (Susiana *et al.,* 2018; Meng*et al.,* 2019).

The effect of adding *Trichoderma* isolates as conidial suspensions to the soil surface had greater effect than root dipping for potato plant growth under glasshouse conditions. It could be due to the strong ability of *Trichoderma* to convert soil nutrients and then dissolution of nutrients to be useful for increasing plant growth (Halifu *et al.* 2019). The root dipping method was less effective technique for plant growth than adding spores to the soil because spores can only penetrate the root through root hairs or epidermis directly without using the soil nutrients.

In the colony diameter assay of AG-3PT isolate on the studied types of soil, the colony growth rate of the pathogen was highly inhibited in Dorrigo soil, whereas Trevenna soil and sand-potting mix gave higher colony radius in comparison with other type of soils, probably because these soils differ in physical properties and available nutrients which can play significant role in the growth of soil microorganisms (Wimpenny, 1979; Kreft*et al.,* 1998). Results indicate that Dorrigo soil reduced the pathogen growth which is consistent with *Trichoderma* isolates as biocontrol agents isolated from this area protecting the infection of potato by AG-3PT isolate of the pathogenic fungus.

In greenhouse trials, *Trichoderma* isolates as biocontrol agents was reduced the disease severity of stem canker and black scurf and showed different levels of brown canker on the stem and at the same time there were no sclerotia (scurf) appeared on tubers. The biocontrol agents that obtained from the root of potato roots and rhizosphere zone were not equally efficient in suppressing the incidence of disease. All *Trichoderma* isolates increased potato growth indicators. T5, T6 and T8 were protected plants. From the -infection by, the pathogen and increased vegetative growth of plants, especially T8 which had strong promotion of growth than other isolates. There was no brown canker for both trials either vermiculite or sand potting mix; however, other *Trichoderma* isolates not prevented potato from the infection completely. This result is in agreement with a study conducted by Alshimaysawe (2018) who mentioned that *Trichoderma* strains had the ability to protect or diminish disease severity or incidence of pathogen strain AG-3PT. Hence, it may occurred due to the ability of *Trichoderma* isolates for establishing on the root surface of potato plant in addition to other mechanisms such as hyper parasitism, antagonism, stimulation of systemic resistance, endophytic competence, antibiotic production and competition for space and nutrients (Nusaibah and Musa, 2019). *R. solani* diminished the vegetative growth of plants and tuber yield, and caused disease symptoms on the tubers of potato.

CONCLUSION

R. solani AG-3PT, the fungal pathogen of stem canker and black scurf diseases on potato is can be found in different types of soil with different levels of population density and it is widespread globally. Studies have been shown that the pathogen reduces all the growth parameters and yield under glasshouse or field conditions. Results of current study demonstrate that treatments with T8 and T5 made the strongest inhibition to the pathogen growth in both dual culture and culture filtrate methods. Moreover, T5, T6 and T8 treatments were eliminated or prevented the fungal disease, promoted plant growth and tuber yield. Further work need to be done on these biocontrol strains in the field to confirm of using them commercially.

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