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Biological Control of Potato Bacterial Wilt Diseases

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Abstract

Potato (Solanum tuberosum L.) is considered one of the four major and important food crops around the world. This study planned to control bacterial wilt disease of potato using some bio-agents which isolated and identified from potato plant soil. Twenty isolates were selected and identified as the following three isolates belonging to Streptomyces spp. (Streptomyces antibioticus (SA1), S. albus (SA2), and S. mutabilis (SM1); five isolates belong to pseudomonas species (Pseudomonas fluoresces (PsF1), P. aeruginosa (PsA1), P. putida (PsP1), P. alecaligones (PsA2) and P. pseudoalcaligones (PsP2)) and twelve isolates were belonging to Bacillus spp. (Bacillus subtilis (BS1-8), B. cereus(BC1), B. badius (BB1-2), B. pumilus (BP1). In vitro, these isolates were examined against the growth R. solanacearum bacterium, where some isolates (BS3, PsF1, BS8, BS6, SM1, BS5, and BS4) were the most effective compared with other isolates. Bacillus subtilis (BS8); Pseudomonas fluorescence (PsF1) and Streptomyces mutabilis (SM1) isolates were selected as bio-agents to control potato bacterial wilt disease under in vivo condition, where these isolates led to reduced disease severity and to increase potato yield compared with the control. The application of bio-agents as drench treatment was more effective than tuber treatment, and isolates of S. mutabilis (SM1) and B. subtilis (BS8) were more effective than Pseudomonas fluorescence (PsF1) isolate.

Keywords: *Potato, Solanum tuberosum L., Bacterial wilt disease, Bio-agents, Ralstonia solanacearum, Biological control*

1. Introduction

Ralstonia solanacearum, the causative of bacterial wilt (brown rot of potato) disease is widely spread in tropical and subtropical areas. This bacterium is a unique plant pathogen according to its economic and scientific value Mansfield et al., (2012). Brown rot is the second most important tropical and subtropical potato disease after late blight. Champoiseau et al., (2009). Brown rot disease, the most important potato bacterial disease in Egypt, was announced many years ago. Khairy et al., (2021). It is challenging to discover an effective method of controlling potato bacterial wilt because of the pathogen's persistence and ability to spread using irrigation water, soil, surface water, agricultural machinery, and infected organic material Janse,)1996 (and Tan et al., (2016).

Biological control is supposed to preserve environmental quality by minimizing dependency on chemical inputs and maintaining sustainable management practices Bara and Jeffries, (1995). Soesanto et al., (2013) reported the successful antagonistic Bacillus sp. and Pseudomonas fluorescens isolated from the rhizosphere to control bacterial wilt on potatoes by delaying the incubation period suppressing disease intensity decreasing the final pathogenic population and inducing plant resistance.

Plant growth-promoting Rhizobacteria (PGPR) are recognized for growth promotion and disease Control Maji and Chakrabarty, (2014) and Apastambh et al., (2016). Many chemical, biological, and physical tools were used to control R. solanacearum bacterium. Biological control agents are hopeful eco-friendly tools alternative to pesticides to manage diseases that are beneficial in protecting crop plants and safeguarding food as well He et al., (2021). Suppression mechanisms are typically attributed to the antibacterial metabolites produced by biological control agents or those present in natural products and increasing host resistance to the pathogen. Nion and Toyota, (2015).

Cultural and biological control of bacterial diseases were alternative solutions with some promising success Michel and Mew, (1998) Ran et al., (2005). Bacterial wilt disease cannot be controlled by chemical means. Up till now, no effective chemical products are available for controlling "bacterial wilt" (R. solanacearum). Among the several methods of disease management, biocontrol plays an important role in disease control and may help the development of alternative management measures being integrated with other practices for effectiveness. Control and for minimizing environmental Pollution due to the use of chemical pesticides Lwin and Ranamukhaarachchi, (2006) and Achari and Ramesh, (2014). The present work was planned to isolate and identify some antagonistic microorganism and their effects on the growth of R. solanacearum, the severity of Potato bacterial wilt, and potato yield under artificial inoculation conditions.

2. Materials And Methods

Isolation and identification of potato bacterial wilt pathogen

Samples of potato tubers with typical disease symptoms of bacterial wilt were collected from different districts in some governorates. Isolation procedures were carried out using tetrazolium chloride (TZC) medium and South Africa (SMSA) described by Engelbrecht (1994) and Elphinstone (1996). Collected isolates were identified using cultural, and morphological characteristics, and biochemical and physiological tests were studied using the methods described by Schaad et al., (2001); and McCarter (1976). Immunofluorescence antibody staining (IFAS) test was applied to confirm their identification in Potato Brown Rot Project, Dokki, Giza, Egypt (2022). Positive isolates were inoculated in tomato seedlings to verify their pathogenic potential (Janse, 1988 and Wenneker et al., 1999).

Isolation of antagonistic microorganisms from potato rhizosphere

Ten grams of potato plants rhizosphere soil were collected from infested fields with bacterial wilt disease. Each sample was placed in flask (250 ml) containing 90 ml of sterile distilled water and shaken for 30 min. using horizontal shake. Serial dilution was prepared and one ml of 104, 105 and 10^7 dilution were poured on nutrient agar medium for Bacillus spp according to (Lelliolt and Stead, 1987), on king`s B medium for Pseudomonads fluorescents spices (King et al., 1954) and on starch nitrate agar medium for actinomycetes spices (Shirling and Gottleib, 1966). These plates were gently rotated and incubated at 27□1°C for 3, 7 and 14 days, respectively. Selected colonies were transferred from mixed culture of the plates onto respective agar plates for purification by streak plate technique and the plates were incubated at 27□ 1°C for 2-7 days. After purification, plates containing pure cultures were stored at 4°C until further studies. Four plates were used as replicates for each treatment.

Identification of antagonistic bacteria and actinomycetes isolates

Identification of the antagonistic bacteria (Bacillus spp and Pseudomonas species) was made through morphological as well as biochemical characteristics according to the method described by Fahy and Persley (1983). Actinomycetes isolates were identified as described by Cross (1989); Locci (1989) and Williams, et al. (1989). All identification procedures were carried out in the regional center of Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

The antagonistic effect of isolated bacteria and actinomycetes against R. solanacearum bacterium, in vitro: The antagonistic effect of bacteria and actinomycetes against the growth of R. solanacearum bacterium, using the technique described by Madigan, et al. (1997). The plates were prepared and inoculated with the tested bacteria or actinomycetes cultures by a single streak of inoculum at a certain distance (3 cm) from the center of the petri dish and incubated at 27±1°C for 4 days for actinomycetes and 2 days for the tested bacteria. Later, the plates were seeded with R. solanacearum

by a single streak starting from the center of the plate and directed to the tested organism (bacteria or actinomycetes) at a 90□ angle to the tested organism. Antagonism was measured by determining the length of the inhibition zones (mm), and different inhibition zones were recorded. The antagonistic effect of isolated bacteria and actinomycetes against R. solanacearum bacterium, in vivo:

The virulent isolate of Ralstonia solanacearum (RS3) isolated from an infected potato tuber was grown on sucrose peptone agar medium for 48 h at 28°C. Bacterial growths was suspended in sterilized tap water and the population density of the bacterium was adjusted to give 6 x 108 colony-forming units (CFU/ml) using a spectrophotometer (OD600 = 0.3). The sterilized clay soil was distributed in sterilized clay pots 30 cm in diameter (7 Kg soil/pot) and separately infested with the bacterial inoculum to give a final concentration of 107 CFU/g dry soil. The infestation procedure was made five days before planting according to Michel and Mew (1998). Antagonistic isolates were grown on an NA medium for 48 h for (bacteria) and 7 days for (actinomycetes). A concentration of 109 CFU/ml was prepared for each antagonistic bacterium and actinomycetes using a Spectrophotometer (OD600= 1) according to Ciampi and Sequeira (1980). Antagonists were applied singly as soil or tuber treatment. In soil treatment, each pot was treated with 500 ml of the antagonistic isolate at the time of sowing. Meanwhile, in tuber treatment, potato tubers (cv, Nicola) were soaked in the suspensions of the antagonistic bio-agents plus 0.1% methylcellulose (1:1 v/v) for 60 minutes before sowing in infested soil. Four pots were used as replicates for each treatment. Twelve replicates with only the pathogen were used as control treatments. All pots were kept under outdoor conditions.

Disease assessment

Disease severity was assessed after 70 and 90 days after planting. The severity of bacterial wilt was calculated as a percentage of sprouts showing wilt symptoms per plant and the disease index (%) was calculated from disease rating for individual plants (Kempe and Sequeira 1983) using the scale based on the visual observation of the percentage of foliage wilt (0 = no visible symptoms, 1 = 1- 25% wilt, 2= 26- 50%, 3 = 51-75% wilt, 4= 76- 100% wilt and 5 = dead plants. The percentage of disease index (DI%) was calculated by the following formula:

$$DI = \frac{\sum RT}{5N} \times 100$$

Where: T = Total number of plants with each category.

R = Disease severity scale (R = 1, 2, 3, 4 and 5)

N = Total number of tested plants.

Disease reduction percentages (DRP) were calculated from disease index using the following formula:

$$PDR = x\frac{DIck - DItr}{DIck} \times 100$$

Where: $\mathbf{DI_{ck}}$ **=** Disease index in check treatment.

$\mathbf{DI_{tr}}$**=** Disease index in treated treatment.

Statistical analysis

The determination of statistical difference was based on a comparison of the disease severity and the yield. First, the initial conditions of validation were tested to determine which approach, parametric or nonparametric, should be used. If conditions of normal residues distribution and homoscedasticity were fulfilled, a parametric approach based on an ANOVA followed by pairwise multiple comparisons (ad hoc test of Duncan's method) was performed. Otherwise, a nonparametric approach based on a Kruskal-Wallis test was used. The analyses were performed, using the Excel Add-in XLSTAT software program (Addinsoft 2021).

3. Results and Discussion

Isolation and identification of the causal organism from infected potato tubers

Collected isolates were identified as *Ralstonia solanacearum* bacterium according to morphological, cultural, physiological, and biochemical characteristics. This identification was performed with the immunofluorescent staining system in Potato Brown Rot Project. (fig. 1).

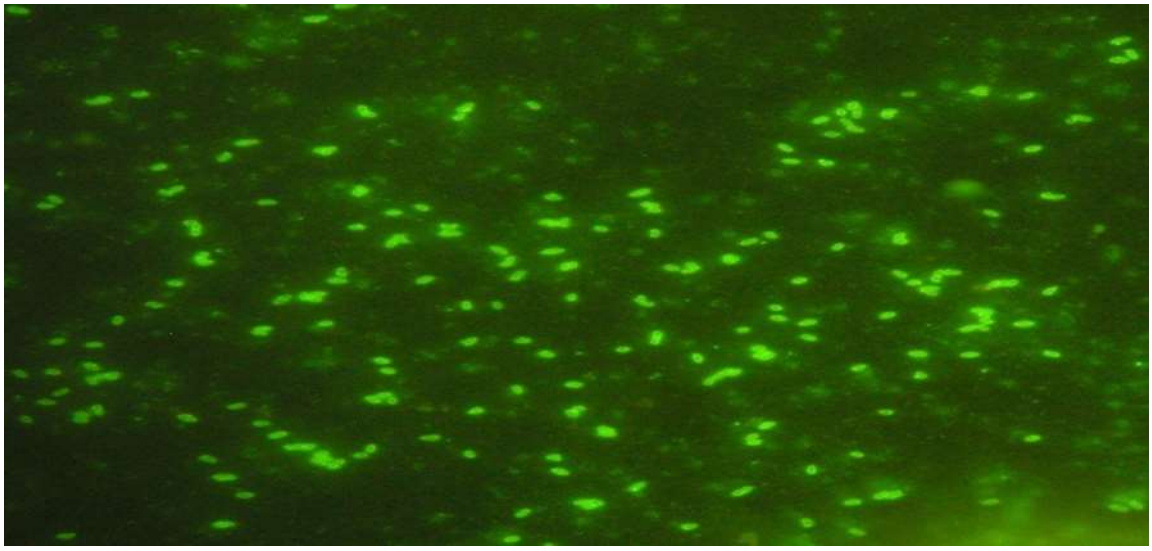

Fig. (1): Cell morphology of *R. solanacearum* in the serological immunofluorescent antibody staining (IFAS) test where cells are short rods and stained evenly as bright green fluorescent under an IF microscope

Identification of antagonistic microorganisms from soil potato plants

Twenty isolates were selected and identified according to cultural, morphological, and biochemical properties (table 1-3). Data in Table (1) revealed that three isolates belonged to *Streptomyces* spp, where these isolates were identified as the following *Streptomyces antibioticus* (SA1), *S. albus* (SA2), and *S. mutabilis* (SM1). Also, five isolates were belonging to *pseudomonas* species, where these isolates were identified as the following *Pseudomonas fluoresces* (PsF1), *P. aeruginosa* (PsA1), *P. putida* (PsP1), *P. alecaligones* (PsA2) and *P. pseudoalcaligones* (PsP2) (table 2). But twelve isolates belonging to *Bacillus* spp as the following *Bacillus subtilis* (BS1-8), *B. cereus* (BC1), *B. badius* (BB1-2), *B. pumilus* (BP1) (table 3).

Table (1): Characterizations of isolated antagonistic actinomycetes (*Streptomyces* spp) and their ability to carbon sources utilization

Characteristic	Code isolate of actinomycetes		
	SA1	SA2	SM1
Sporphore morphology	Straight to flexible	Straight to flexible	Straight to flexible
Spore surface	smooth	spiny	Smooth
Growth	Well	Well	moderate
Color of aerial mycelium	Gray	Grayish brown	white
Color of growth	Brownish gray	brownish yellow	Non-distinctive
Soluble pigment	Negative	Negative	Negative
Cellulose decomposition	negative	Negative	Negative
Gram stain	+	+	+
Sporulation	+	+	+
Cell shape	Hyphae	Hyphae	Hyphae
KOH test	-	-	-
Motility	-	-	-
d. Glucose	+	+	+
D. Fructose	+	-	+
D. xylose	+	-	+
L. rhaminose	+	+	+

Rafinose	-	+	-
Sucrose	-	+	+
Glycerol	+	+	+
Starch	+	+	+
Identification	*S. antibioticus*	*S. albus*	*S. mutabilis*

Table (2): Morphological and biochemical Characteristics of antagonistic bacteria (*Pseudomonas* spp.)

Characteristic	Code isolate of bacteria				
	PsF1	**PsA1**	**PsP1**	**PsA2**	**PsP2**
Cell shape	Short rods	Short rods	Short rods	Short rods	Short rods
Gram staining	-	-	-	-	-
Sporulation	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	+
Levan production	+	-	-	+	+
Gelatin hydrolysis	+	+	+	+	+
Oxidase	+	+	-	+	+
Catalase	+	+	-	-	+
Starch hydrolase	-	-	-	-	-
Indole production	-	-	-	+	+
Pigment on KB medium	+	+	+	-	-
Utilization of:					
Glucose	+	+	+	-	-
Trehalose	+	-	-	-	-
D-Mannose	+	-	+	+	+
Sucrose	+	-	-	+	+
Identification	*P. fluorescens*	*P. aeruginosa*	*P. putida*	*P. alcaligenes*	*P. pseudoalcaligones*

Table 3: Morphological and biochemical Characteristics of antagonistic bacteria (*Bacillus* spp.)

Characteristic	Code isolate of bacteria											
	BS1	**BS2**	**BS3**	**BS4**	**BS5**	**BS6**	**BS7**	**BS8**	**BC1**	**BB1**	**BB2**	**BP1**
Cell shape	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+
Sporulation	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+
Levan production	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	-	+

Oxidase	-	-	-	-	-	-	-	-	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolase	+	+	+	+	+	+	+	+	+	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of:												
Glucose	+	+	+	+	+	+	+	+	+	-	-	-
Arabinose	+	+	+	+	+	+	+	+	-	-	-	-
Identification	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. cereus*	*B. badius*	*B. badius*	*B. pumilus*

Effect of isolated microorganisms on the growth of *R. solanacearum, in vitro*

Twenty isolates of antagonistic microorganisms were examined against the growth *R. solanacearum* bacterium, *in vitro* (fig. 2). Five isolates of *Bacillus subtilis* (BS3, BS8, BS6, BS5, and BS4); one isolate of *Pseudomonas fluoresces* (PsF1) and one isolate of *Streptomyces mutabilis* (SM1) was the most effective to inhibited the growth *R. solanacearum* bacterium, where the diameter of inhibition zone was 10.9, 10.9, 10.7, 10.4, 10.3, 10.9 and 10.6 mm, respectively. Meantime, three isolates of *B. subtilis* (BS1, BS7, and BS2); one isolate of *B. cereus* (BC1); three isolates of *Pseudomonas* spp (*P. alcaligenes* (PsA2); *P. pseudoalcaligones* (PsP2) and *P. aeruginosa* (PsA1)) and two isolates of *Streptomyces* spp, (*S. antibioticus* (SA1) and *S. albus* (SA2)) were moderately effective, where the inhibition zone was 5.9, 5.5, 5.3, 5.6, 5.4, 5.4,5.1, 5.9, and 5.4 mm, respectively. Meanwhile, three isolates of *Bacillus* spp. (*B. pumilus* (BP1) and two isolates of *B. badius* (BB1, 2)) and one isolate of *P. putida* (Psp1) were less effective, where the inhibition zone was 0.9, 0.5, 0.3, and 0.5 mm, respectively.

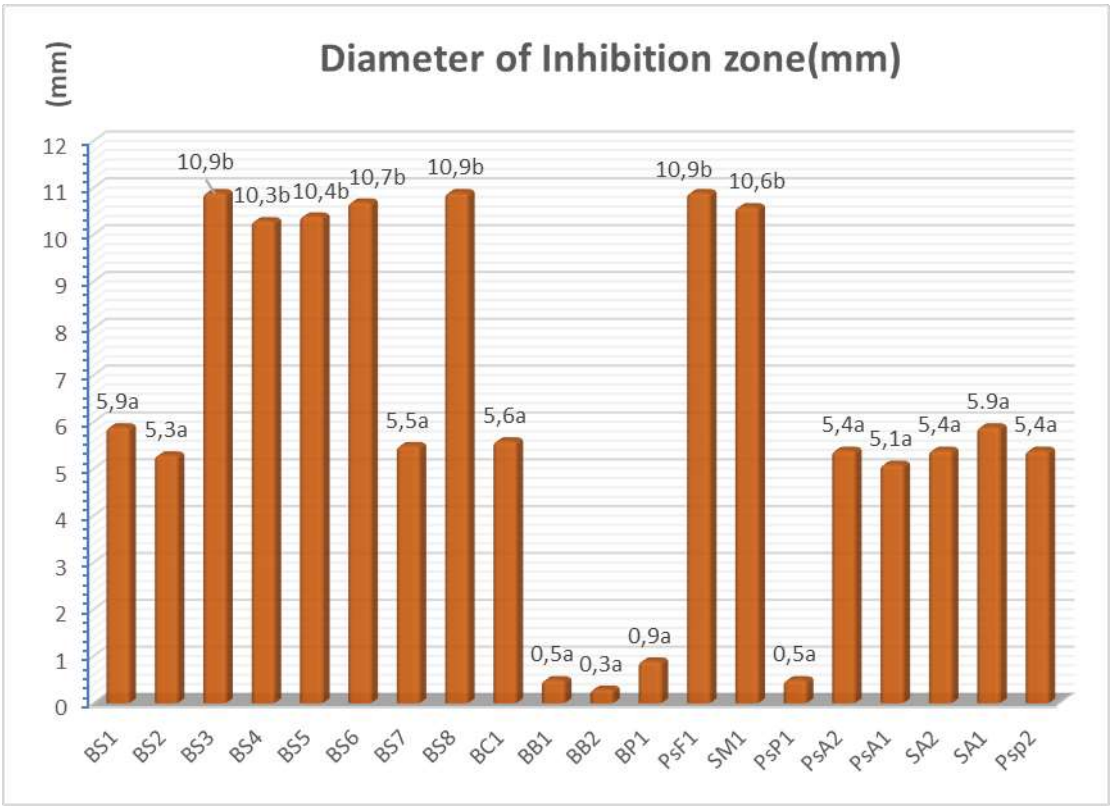

fig. (2): Effect of some bio-agents on the growth of potato bacterial wilt bacterium (*Ralstonia solanacearum*), *in vitro*

Effect of some bio-agents on bacterial wilt disease of potato and the total yield

Bacillus subtilis (BS8); *Pseudomonas fluorescens* (PsF1) and *Streptomyces mutabilis* (SM1) isolates were applied as bio-agents to control potato bacterial wilt disease under artificial inoculation conditions (fig. 3-7). Data in figures (3 and 4) showed that examined bio-agents decreased the severity of bacterium wilt disease in potatoes compared with the control treatment. Likewise, the application of bio-agents as drench treatment was more effective than tuber treatment against the disease, where efficiency treatment ranged from 9.2 to 16.4% and from 5.8 to 15.2%, respectively. While, isolates of *S. mutabilis* (SM1) and *B. subtilis* (BS8) were more effective to control the disease than *Pseudomonas fluorescens* (PsF1) isolate, where efficiency treatment was 5.4 - 16.4, 5.0 -16.0 and 5.8 -13.6 %, respectively.

However, results in figure (5-6) appeared that the application of *B. subtilis* (BS8); *S. mutabilis* (SM1), and *P. fluorescens* (PsF1) isolates as bio-agents led to reducing the severity of infected tubers and increased potato yield (number and weight tubers) compared with the control treatment. While, drench treatment was more effective than tubers treatment, where efficiency for controlling infected tuber was 10.3 - 27.5% and 3.3 - 22.4%, and potato tubers number was 2.5 - 3.3 for 2.3 -3.0 tuber/plant and 44.30 - 65.05 and 40.64 - 60.60 g/plant, respectively. Meanwhile, *B. subtilis* (BS8) and *S. mutabilis* (SM1) isolates were more effective than *Pseudomonas fluorescens* (PsF1) isolate, where efficiency to control infected tuber was 22.4 - 27.5; 16.3 - 21.8% and 3.3 -10.3 % and the yield were 3.0 - 3.3; 2.5 - 2.7 and 2.3 - 2.5 tuber/plant and 60.60 - 65.05; 49.53 - 52.56 and 40.64 - 44.30 g/plant, respectively.

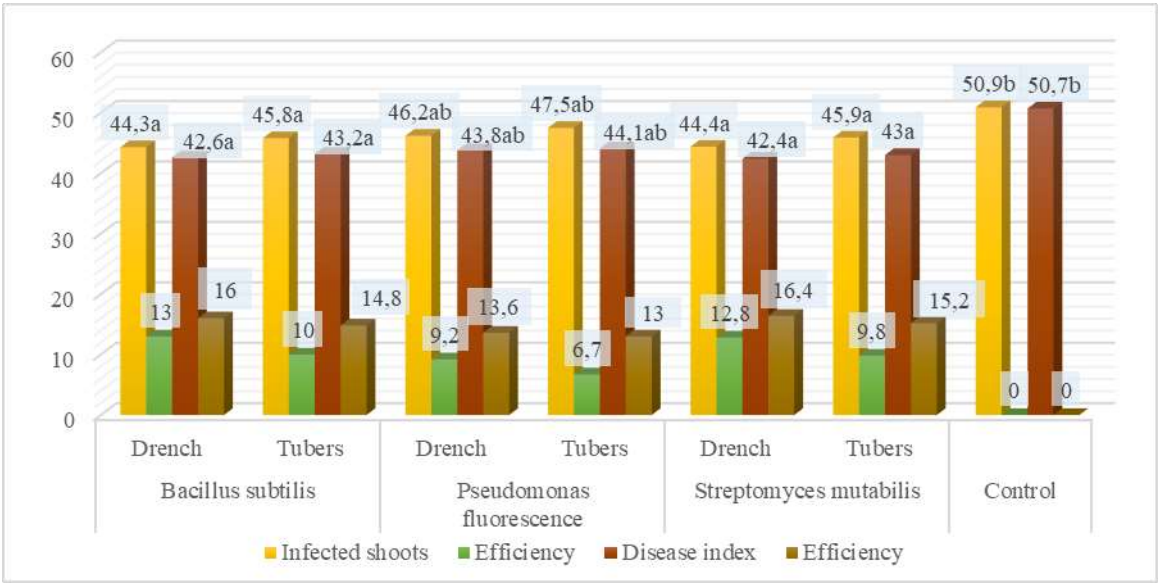

Fig 3: The efficiency of bio-agents on the severity of potato bacterial wilt disease, using two methods of treatment, after 70 days from artificial inoculation

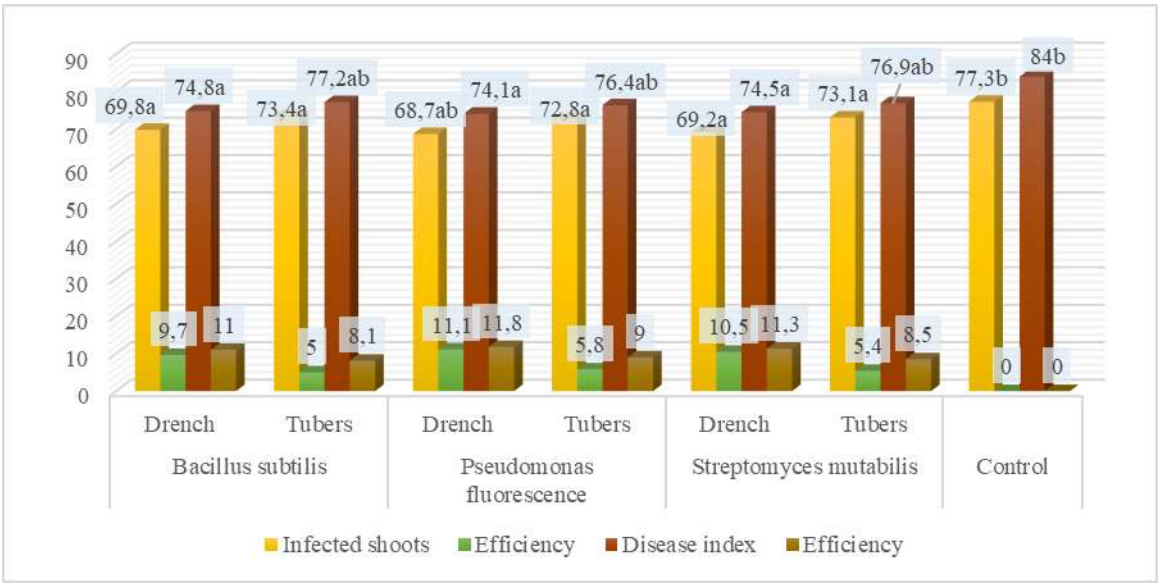

Fig 4: The efficiency of bio-agents on the severity of potato bacterial wilt disease, using two methods of treatment, after 90 days from artificial inoculation

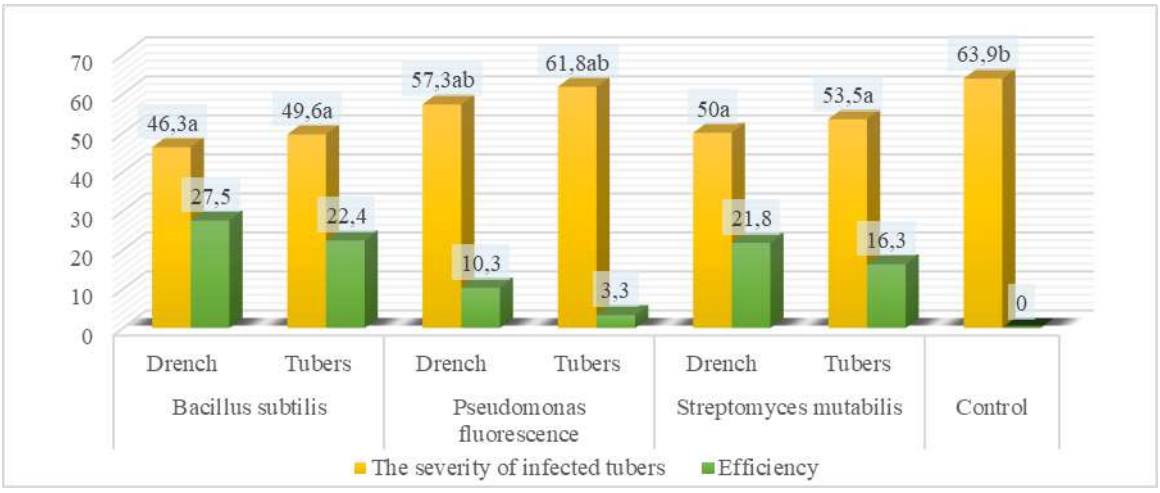

Fig 5: The efficiency of bio-agents on the severity of infected tubers (%), using two treatment methods, after 105 days from planting, under artificial inoculation

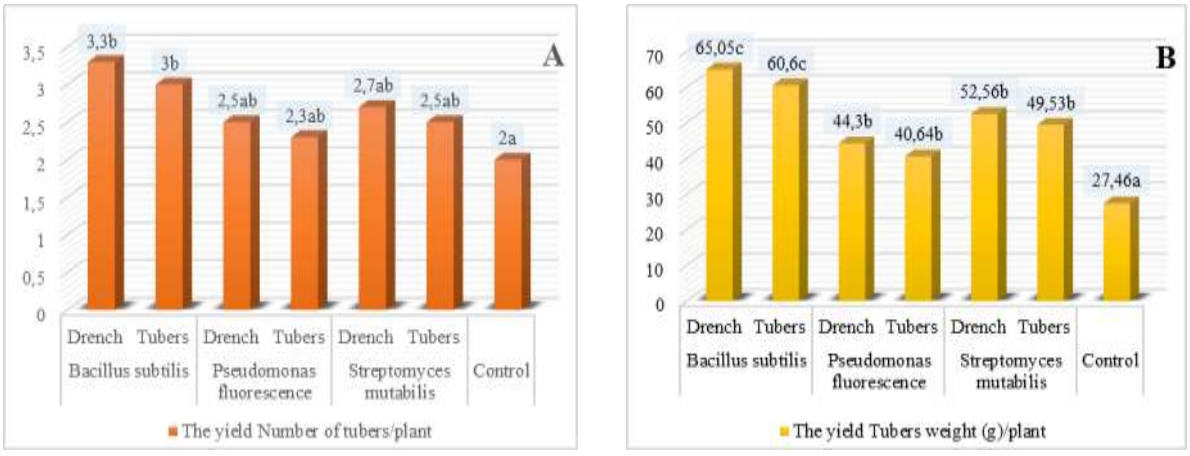

Fig 6: Effect of some bio-agents on the yield (A. Number of tubers, B. The weight of tubers/plant), using two treatment methods, after 105 days from planting, under artificial inoculation

Twenty isolates of antagonistic microorganisms were isolated and identified from rhizosphere potato plants as the following: Three isolates belong to Streptomyces species (*Streptomyces antibioticus* (SA1), *S. albus* (SA2), and *S. mutabilis* (SM1)). Also, five isolates belong to *pseudomonas* species (*Pseudomonas Fluorescent* (PsF1), *P. aeruginosa* (PsA1), *P. putida* (PsP1), *P. alecaligones* (PsA2) and *P. pseudoalcaligones* (PsP2). Meanwhile, twelve isolates belong to *Bacillus* species (eight isolates of *B. subtillis* (BS1-8), two isolates of *bacillus badius* (BB1-2), *B. cereus* (BC1) and *B. Pumilus* (BP1). Soesante *et al.,* (2013) mentioned successfully isolated antagonistically *Bacillus* spp. and *Pseudomonas fluorescens* from rhizosphere Potato plants with infection by bacterial wilt disease and delaying incubation Period. Zhu *et al.,* (2021) mentioned that *Bacillus* species have a long history in biocontrol and crop growth-promoting applications. *Pseudomonas aeruginosa* isolate showed higher antifungal activity and a lower minimum inhibitory concentration Shiakh *et al.,* (2014).

Some plant-associated bacteria are classified as beneficial microorganisms based on their effects on plant performance, and free-living plant growth-promoting rhizobacteria (PGPR) thrive freely in the rhizosphere soil (Labuschagne *et al.,* (2011), Shaikh *et al.,* (2018)). Antagonistic bacteria from the genera Pseudomonas, Bacillus and Streptomyces, account for the majority of rhizosphere microorganisms commonly used in biocontrol. Ciancio *et al.,* (2019).

Isolates of antagonistic microorganisms appeared differential effective against the growth of *R. solanacearum* bacterium *in vitro*. *Bacillus subtilis* (BS8), *P, fluorescent* (PsF1), and *S. mutabilis* (SM1) isolates were the most effective compared with other isolates. These isolates were selected as bio agents to control bacterial wilt disease of potato, under artificial inoculation conditions. Ali *et al.,* (2013) found that plant growth-promoting rhizobacteria (PGPR) excretion different antibacterial substances in the culture medium. These substances reduce the growth of the bacteria and formed an inhibition zone. Balabel *et al.,* (2013) applied some antagonistic species against five isolates of *Ralstonia solanacearum* bacterium which showed varying antagonistic potential. Abd El Rahman and Shaheen (2016) found that *Bacillus subtilis*, *B. cereus*, *Pseudomonas fluorescens*, and *Rhizobium phaseoli* affect the growth of *Ralstonia solanacearum*. *In vitro*, where *B. subtilis* was the most effective, and *P. fluorescens* had the most growth potential and great inhibition on King's B medium. Arfaoui *et al.,* (2006) and Aliye *et al.,* (2008) reported that antagonism of *Bacillus* Spp, *P. fluorescens* and *Rhizobium* spp against the different plant pathogens, *In vitro*.

Selected antagonistic microorganism isolates decreased the severity of potato bacterial wilt and increased potato yield compared with the control. Likewise, the application of bio agents as soil drench treatment was more effective than their application as tubers treatment. *Streptomyces mutabilis* (SM1) and *Pseudomonas fluorescens* (PsF1) isolates were highly effective on disease severity and the yield compared with *Bacillus subtilis* (BS8) isolate. Wagih (1991) and Sunaina *et al.,* (1997) revealed that potato bacterial wilt (brown rot) disease reduced significantly after bacterization of healthy tuber potatoes with *Bacillus cereus*, *B. subtilis*, and with a virulent strain of *R. solanacearum* under field conditions. Aspiras and Cruz (1985) mentioned that *P. fluorescens* was highly effective in reducing bacterial wilt in tomato and potato under experimental conditions by aggressively colonizing the roots of young plants and preventing entry of *R. solanacearum.* Buyer, and Leong, (1986) and Ran *et al.,* (2005) found that inhibition of the pathogen by *P. fluorescens* was attributed to the siderophore pseudobactin, depriving rout colonizing microorganisms, including plant pathogens of Fe^{3+} and inducing systemic resistance.

4. Conclusion

Application of *Bacillus sp*., *B. subtilis*, and *P. fluorescens* effectively controlled brown rot pathogen and increased potato yield Atia *et al.,* (2010). Rosyidah *et al.,* (2013) reported that a single application of *P. fluorescens* or combined application of *Streptomyces* spp and *Trichoderma viride* plus *Streptomyces* spp was capable reducing disease incidence, disease intensity, and, the population of *R. solanacearum*. Bacterial isolates' ability to produce antagonistic and growth-promoting substances modified their ability to increase plant growth and inhibited other organisms. Compant *et al.,* (2005). perez-Garcia *et al.,* (2011) mentioned that promising achievements in terms of biological control have emerged, especially after the successful use of certain antagonistic biocontrol agents (BCAs), in particular *Pseudomonas* spp *Bacillus* spp *Burkholderia* spp and *Trichoderma* spp against pathogens causing foliar and soil-borne diseases. Alizadeh *at el.,* (2013) showed that biopesticides are available

inside the rhizosphere to combat pests and microbial diseases due to the close connection of root-colonizing probiotic microorganisms with plant host cells.

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