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Biological Control of Onion White Rot Disease Using Different *Bacillus* spp.

[1]Afaf, Z.A. El-Meneisy, [2]Samah M. Abdelaziz and [2]Rabaa Y.K. Yaseen

[1]Plant Pathology Department, Faculty of Agriculture, Ain Shams University,
P.O. Box: 68, Hadayek Shobra 11241, Cairo, Egypt
[2]Fertility and soil Microbiology Department, Desert Research Institute,
P.O. Box: 1, El-Matareya 11753, Cairo, Egypt

Abstract: White rot is a serious disease of *Allium* spp. caused by the soil-borne fungus *Sclerotium cepivorum*. Among sixty eight bacterial isolates tested against *Sclerotium cepivorum*, three isolates gave best antagonistic effect. These isolates were identified as *Bacillus subtilis, Bacillus amyloliquefacienes* and *Bacillus velezensis* depending on morphological, biochemical characters and on 16 s rRNA sequencing. *B. amyloliquefacienes* and *B. velezensis* were more active to solubilize phosphate and produce Indol acetic acid (IAA), siderophores, protease more than *B. subtilis*. *B. velezensis* was the most effective isolate to reduce pathogen growth in vitro. In pots experiment, transplants treated with different bacterial bioagent indicated significant reduction of disease index. The most efficient bacteria was *B. velezensis* followed by *B. subtilis* and *B. amyloliquefacienes* compared with infected control and fungicide treatment. High activity in polyphenol oxidase and peroxidase was detected in treated plants compared with control.

Key words: *Sclerotium cepivorum* · Biocontrol · White rot · *Allium* · Onion · *Bacillus subtilis* · *Bacillus amyloliquefacienes* and *Bacillus velezensis*

INTRODUCTION

Sclerotium cepivorum Berk, is considered as the most aggressive pathogen of *Allium* found at where *Allium* species are grown [1]. This pathogen can cause great damage and losses reached to 100% [2, 3]. *S. cepivorum* is difficult to control because its dormant sclerotia can stay in the soil for more than 15 years [4]. Seed treatment by chemical fungicides considered the most common control strategy for *S. cepivorum* but for a long time it failed and become non effective because of the appearance of pathogen resistant races [5]. The other suggested control methods are including soil solarization, fumigation and application of biological control [4, 6]. Several fungal and bacterial antagonists have proved to control different plant pathogenic fungi [7]. Best results to control white rot of onion were recorded by using *Trichoderma harzianum, T. Koningii, T. asperellum, Talaromyces flavus, Bacillus subtilis* [6, 8]. Using microbial antagonists as a bioagents is considered a suitable ecologically way to substitute chemical fungicides. This study aimed to use an effective and safe method for control white rot of onion as an alternative method instead of using a harmful chemical fungicide.

MATERIALS AND METHODS

Isolation of the Pathogen: Samples of onion plants that showing white rot symptoms were obtained from Al Kanater Al Khairia, Governorat of Kaliobiya. Infected bulbs were cleaned with water, cutting into small parts then sterilized with sodium hypochloride (0.5%) for about two minutes, the excess of sodium hypochloride was removed by washing in sterilized distilled water. Samples were dried well using sterilized filter papers and then plated on potato dextrose agar (PDA) medium then incubated for 5 days at 20°C. hyphal tip technique was used to purifying cultures [9]. The slants of pure cultures were maintained at 5°C for further studies.

Corresponding Author: Afaf Z.A. El-Meneisy, Plant Pathology Department, Faculty of Agriculture, Ain Shams University, P.O. Box: 68, Hadayek Shoubra 11241, Cairo, Egypt.

Inoculum Preparation and Pathogenicity: Glass bottles (500 ml) contains barley grains (100g) and water (50 ml) were autoclaved and then inoculated with discs (5 mm diameter) of fungal isolates (10 days-old) and incubated for 25 days at 20°C [10].

Four fungal isolates were inoculated on onion cultivar (Giza 20) for pathogenicity test, under greenhouse conditions. Sterilized pots with 25 cm diameter were filled with a mixture of sterilized clay soil and fungal inoculum (2% w/w). Each one was contained 3 kg infested soil. Pots contained sterile soil were used as control. The soil was kept moistened before transplanting for about two weeks. Three onion seedlings (60 days old) were transplanted in each pot. Disease symptoms were recorded weekly.

Isolation of Antagonistic Bacteria: Samples of healthy onion soil were collected from north west and south west of Minya and south east of El-Monkhafdd areas. Serial dilutions method was used to isolate bacteria from soil samples [11].

***In vitro* Assay:** Sixty-eight bacterial isolates were examined against *Sclerotium cepivorum* on plates of potato dextrose agar (PDA) medium according to Abd-Elrazik *et al*. [12]. Plates of PDA were streaked with bacterial isolates on one side. fungal disc (5 days old culture) were transferred to the other side of plates. Plates inoculated only with a pathogen served as a control. Antagonistic activity was recorded after incubation at 20°C until complete fungal growth of the plates of control. The percentage of reduction in the fungal growth was calculated according to Fokkema [13] as follows:

Reduction in linear growth (%) = (R1-R2|) / R1X 100

where: R1 = the normal growth of control
R2 = the inhibited growth

Identification of Selected Isolates: The molecular identification of selected isolates was carried out at Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza, Egypt. Extraction of Genomic DNA from isolates was carried out according to Ishikawa *et al.* [14].

Phylogenetic Analysis: Sequences of 16S rRNA for the selected isolates were constructed in BioEdit softwar [15]. NCBI database was used to compare the obtained nucleotide sequence through BLAST program (www.ncbi.nlm.nih.gov/blst). Multiplication of nucleotide sequences alliance was performed using BioEdit and Clustal W. Neighbour joining phylogenetic tree was constructed by using Phylip 3.65 [16]. Neighbor joining (NJ) method was used to assemble the phylogenetic tree [17]. The clustering stability of the tree was evaluated by 100 data sets of bootstrap analysis.

Plant Growth Promotion Activities of Selected Isolates
Production of Indole-3-Acetic Acid (IAA): Bacterial isolates were examined for their capability to produce Indole-3-acetic acid according to Patten and Glick [18]. Bacterial isolates were grown on nutrient broth medium contains L-tryptophan for 24 h at 28°C. After incubation, cultures were centrifuged for 15 min at 10, 000 rpm and 4°C then supernatant was collected. Two milliliters of supernatant were mixed with O-phosphoric acid (2 drops) and 2 ml of Salkowski's reagent, tubes with pink colour confirmed IAA production.

Phosphate Solubilization: The ability of bacterial isolates for solubilization of phosphate was determined according to Pikovskaya [19]. Bacterial isolates were spotted separately on plates of Pikovskaya's agar medium (yeast extract 0.5 g/L, Dextrose 10 g/L, Calcium phosphate 5 g/L, Ammonium sulphate 0.5 g/L, Potassium chloride 0.2 g/L, Magnesium sulphate 0.1 g/L, Manganese sulphate 0.0001 g/L, Ferrous sulphate 0.0001 g/L and agar 20 g/L),) and incubated for 3 days at 28°C. colonies had the ability of phosphate solubilization were surrounded with clearing hallo zone.

Production of Organic Acid: Bacterial isolates were examined for their ability to produce organic acid according to Sunstornsuk and Hang [20]. Isolates were inoculated into mineral agar acid indicator medium and incubated for two days for the formation of yellow zone around the bacterial growth.

Hydrogen Cyanide (HCN) Production: Bacterial isolates were assessed for their capabilities to produce HCN qualitatively according to Bakker and Schipper [21]. Plates of nutrient agar medium amended with glycine (4.4 g/L) were streaked with antagonistic bacteria. Sterile filter paper previously immersed in solution of 0.25 % picric acid and 1.25 % Na_2CO_3 was placed in the upper lid of the plates. The dishes were sealed with parafilm and then incubated at 28°C for 48 h.

A change of colour of filter paper from yellow to reddish-brown, brown or light brown was recorded as strong, moderate or weak reaction, respectively.

Production of Siderophores: Siderophores production was determined as stated by Schwyn and Neilands [22]. Bacterial isolates were grown in nutrient broth at 28°C for 24 h. One ml of culture was transferred to iron-free medium and incubated for 5 d at 150 rpm and 30°C.

The medium was extracted with 8-hydroxyquinoline 3% (w/w) in chloroform solution. Siderophore production was observed by change of color from blue to orange using ternary complex Chrome Azurol S as an indicator.

Enzymatic Activity of Selected Isolates: The ability of selected isolates to produce amylase, protease and gelatinase were tested by plate assay method [23].

***In vivo* Assay:** Three bacterial isolates were chosen to study their effect on severity of onion white rot disease, under greenhouse condition. The isolates were grown on nutrient agar medium for 48 hr then collected and suspending in water. the total count of microbial inoculants were adjusted at A_{600} to 10^8 cfu/ml. Onion transplants -60 days old - were immersed in the bacterial inoculants for 12 hr for each isolate [5].

The immersed onion transplants were planted in pots containing infested clay soil (3kg/ pot) and previously watered for two weeks as three plants / pot and five replicates for each treatment. Water- immersed transplants planted in contaminant soil served as a control. Another five replicates were sprayed with folicure (Tebuconazole, alpha-[2-(4chlorophenyl) ethyl]-alpha-(1, 1-dimethylethyl)-1H-1, 2, 4- triazole-1- ethanol) with the recommended dose (25ml/l) as fungicide treatment.

Disease Assessment: Disease severity was estimated after100 days from transplanting using 0-100 scale that reported by Abd El- Moity [10] and Shatla *et al*. [24] as follows:

0 = healthy plants, 25= slightly severe (leaves yellowing, root system reduced), 50 = moderate severe (yellowing, die back of leaves and badly decayed root system), 75 = severe (completely yellowing plant, leaves die back, semi soft rot of scales and roots) and 100 = highly severe (completely dead plants, extensive decayed roots and bulbs).

Determination of Antioxidant Enzymes of Infected Plants: The Activity of plant defense enzymes that affected by bacterial bioagents was determined 60 days after planting.

Polyphenol oxidase assay: Polyphenol oxidase (PPO) activity was evaluated by the increase in O.D. using catechol as a substrate at 420nm. The reaction mixture composed of 1.8 ml of 40 mmol/l catechol dissolved in 0.1 mole/L phosphate buffer (pH 6.5) in the cuvette of spectrophotometer (Spectrophotometer model Hitachi 1009), then 0.4 ml of crude enzyme was added and the absorbance was recorded during 2 min. The activity was expressed in units, where one unit = 0.001ΔA420/min/g fresh weight (FW) [25]. The blank tube was contain mixture of 1.8 ml buffer solution and 0.5 ml of the enzyme solution.

Peroxidase Assay: In this method, purpurogallin formation rate from the reaction between pyrogallol as a donor of hydrogen and hydrogen peroxide as an electron acceptor catalyzed by peroxidase (PO) was determined by spectrophotometer by evaluate the rate of color development at 420 nm during two min [26].

Half ml of the enzyme solution was added into the cuvette of spectrophotometer followed by addition of 1.5 ml phosphate buffer (100 mM) pH 6.0 and 0.1 ml of 5% (w/v) Solution of Pyrogallol. The reaction was initiated by adding 10 ul of hydrogen peroxide solution (0.50%, freshly prepared) and the O.D. was measured during one minute. The blank was prepared without adding hydrogen peroxide.

Unit Definition That amount of enzyme which catalyzes one microgram of purpurogallin produced in 20 seconds at 20°C. at a pH of 6.0.

One unit/ ml = (Delta OD/20sec X 1.71/ 12 X 0.5)X 1000

where
1.71 is the total volume of reaction mixture
12 = Extinction coefficient of 1 mg/ml of purpurogallin at 420 nm
0.5 = Volume (in milliliters) of enzyme solution
1000 is the factor to convert the product from milligram to microgram

Statistical Analysis: Analysis of variance (ANOVA) was carried out according to Steel *et al*. [27]. Duncan's multiple range tests were used to compare treatment means at 0.05level of significant.

RESULTS

Pathogen Isolation and Pathogenicity Test: Isolation process from infected bulbs revealed of four *sclerotium cepivorum* isolates according to cultural and morphological characters. All isolates showed ideal symptoms of white rot disease. Only one isolate was used for further trails.

Isolation of Antagonistic Bacteria: Data presented in Table (1) reveal different locations used for isolation of antagonistic bacteria, number of bacterial isolates and the number of isolates that exhibited anti fungal activities. Among sixty eight bacterial isolates examined against *Sclerotium cepivorum*, thirty six isolates showed inhibition of the fungal pathogen. Three isolates (one isolate from each location) that gave best results were selected and coded B4, B18 and B29.

Antagonistic Efficiency of Bacterial Isolates on *S. cepivorum* Growth: The selected isolates tested against *S. cepivorum* showed high inhibition activities in fungal growth. Data presented in Table (2) and Fig. (1) Indicated high reduction percentage with isolate B29 (84.1 %) followed by isolate B18 (52.6%) and isolate B4 (51.5 %) respectively, compared with control.

Identification of Selected Isolates and Phylogenetic Analysis: Morphological and biochemical characteristics of selected isolates are existing in Table (3). Isolates of B4, B18 and B29 were Gram positive, aerobic, rod-shaped, motile and spore former. The 16S rRNA nucleotide sequence revealed that selected isolates belong to *B. subtilis,* with 96% blast identity, *B. amyloliquefacienes,* with 95% blast identity and *B. velezensis* with 98% blast identity respectively. Phylogenetic tree of the three bacterial strains and closely correlated strains are illustrated in Figs. (2, 3 and 4).

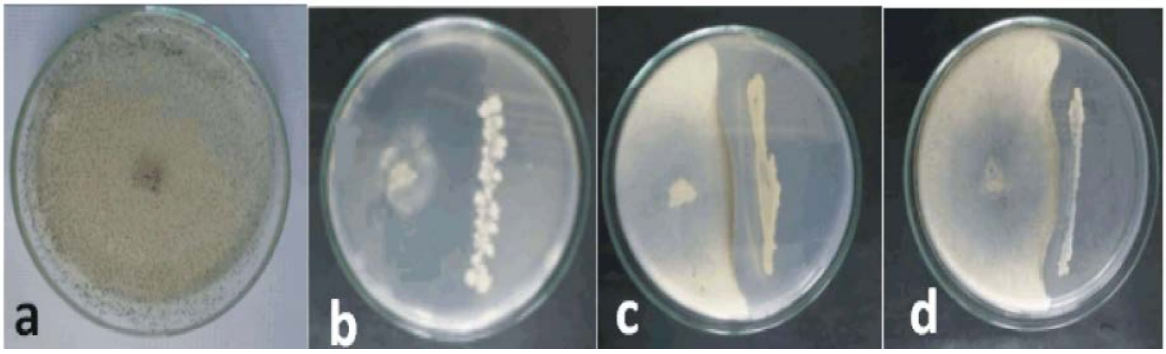

Fig. 1: Growth inhibition of *S. cepivorum* on solid medium by different antagonistic bacterial isolates where, (a) Control (b) B 29 (c) B 18 (d) B 4

Table 1: Isolation of antagonistic bacteria from different location

No	Isolation areas	Number of isolates	Number of positive isolates	Selected isolate
1	North west of Minya	24	15	B 4
2	South west of Minya	27	12	B 18
4	South-east of Al-Monkhafad – EL-Giza	17	9	B 29
Total	----	68	36	3

Table 2: Antagonistic effect of bacterial isolates on *S. cepivorum* growth, *in vitro*

Bacterial isolate	Linear growth (Cm)	Growth reduction (%)
B 4	4.36 b	51.5
B 18	4.26 b	52.6
B 29	1.43 c	84.1
Control	9.00 a	0

Values with a different letter significantly differ at P 0.05 according to Duncan's multiple range tests.

Table 3: Morphological and biochemical characters of selected bacterial isolates

	Bacterial isolate		
Characteristic	B4	B18	B29
Gram staining	+ve	+ve	+ve
shape	Rod	Rod	Rod
Motility test	motile	motile	motile
Spore forming	spore former	spore former	spore former
Methyl red test	-	++	+++
Oxidase test	+	+	+
Catalase test	+	+	+
Arginie dihydrolysis	+	+	+
Utilization of carbon sources			
Glucose	+	+	+
Sucrose	+	+	+
Lactose	-	+	-
Manitol	+	+	+
Glycerol	+	+	+

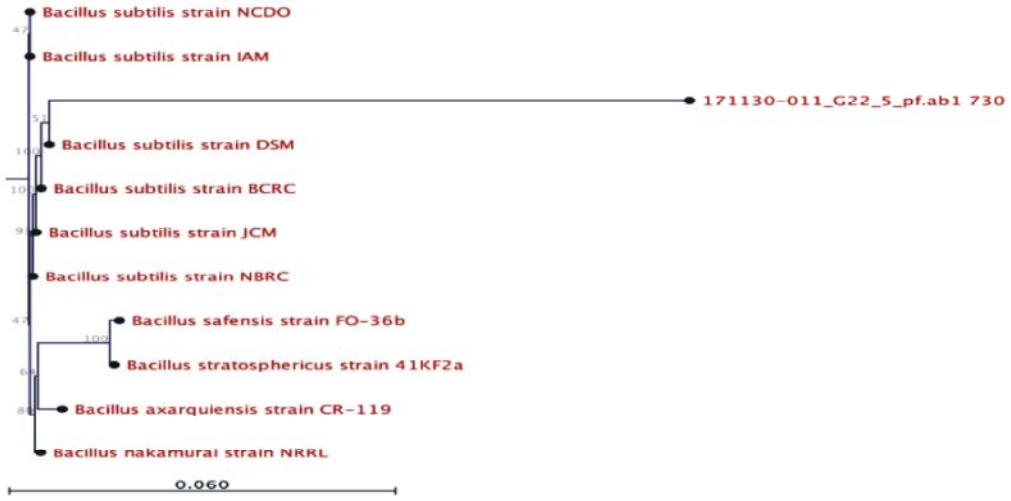

Fig. 2: Phylogenetic tree of partial 16S rRNA sequence for isolate B4. The scale bar represents 10% nucleotide substitutions. Percentages of bootstrap values recovered from 60 trees are presented on the nodes

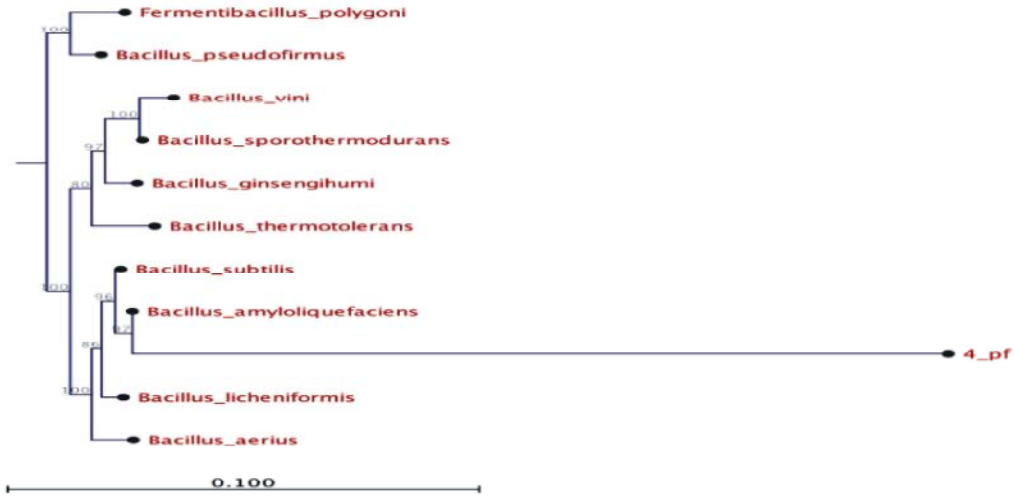

Fig. 3: Phylogenetic tree of partial 16S rRNA sequence for isolate B18. The scale bar represents 10% nucleotide substitutions. Percentages of bootstrap values recovered from 100 trees are presented on the nodes

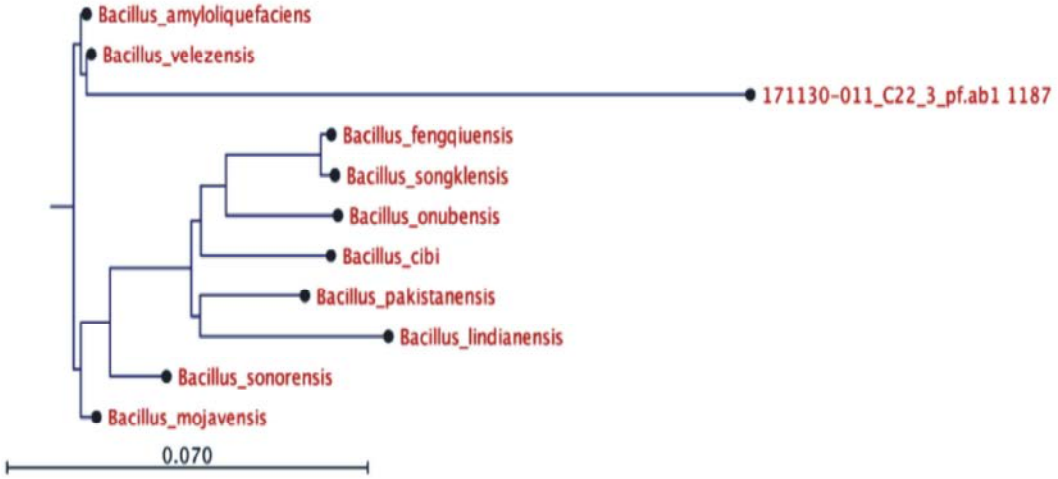

Fig. 4: Phylogenetic tree of partial 16S rRNA sequence for isolate B 29. The scale bar represents 10% nucleotide substitutions. Percentages of bootstrap values recovered from 70 trees are presented on the nodes

Table 4: Activity of bacterial strains for production of growth-promotion and enzymes

	Bacterial isolate		
Characteristic	*B. subtilis*	*B. amyloluqiefacenes*	*B. velezensis*
Indole test	+	++	++
Phosphate dissolving	++	+++	+++
HCN production	-	-	-
Siderophore production	+	+++	+++
Production of enzymes			
Amylase	+	+++	+
Protease	+	+++	+++
Gelatinase	+	+++	+++

Activity of Antagonistic Bacteria for Enzymes and Growth Promotion Production: Results in Table (4) explained that the three bioagent strains have the capability to produce indol acetic acid, siderophores, amylase, protease and gelatinase moreover, solubilizing phosphate but couldn't produce HCN. *B. amyloluqiefacenes* and *B. velezensis* showed high production of previous characters more than *B. subtilis* except of amylase which was highly produced by *B. amyloluqiefacenes* more than the other species.

Enzyme Activity: Activities of peroxidase (PO) and polyphenol oxidase (PPO) enzyme were determined in onion plants under artificial inoculation conditions and assayed during 2 min. It was noted that PPO activity was higher compared with those of PO in all treatments. *Bacillus subtilis* followed by *B. velezensis* and *B. amyloliquefacienes* recorded high activation of PO (11.37, 10.08 and 5.97 U/ ml respectively) compared with infected control (2.22 U/ ml). On the other hand, high activation of PPO was noticed by *B. velezensis* followed by *B. subtilis* and *B. amyloluqiefacenes* (392.5, 587.5 and 637.5 U/ ml respectively) compared with infected control (82.5 U/ ml) as shown in Figures (5 and 6).

Effect of Antagonistic Bacteria on Severity of Onion White Rot, *In vivo*: Under greenhouse condition, Tested bioagents were evaluated for their effects on severity of white rot disease. Disease index and the efficiency percentage of the bioagents were determined Fig. (7). All antagonistic isolates revealed significant reduction in disease index compared with control. *Bacillus velezensis* was the most effective isolate to reduce disease severity by 69.9 % followed by *Bacillus subtilis* (66%) and *Bacillus amyloliquefacienes* (58.4%) compared with control and folicure (56.1%) as fungicide treatment.

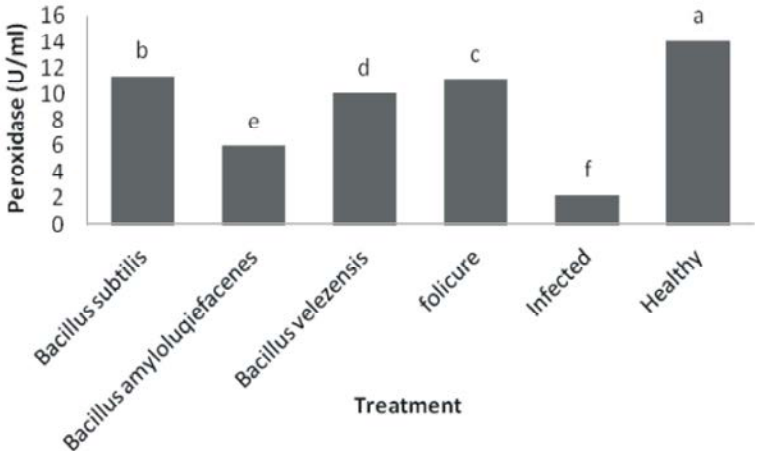

Fig. 5: Peroxidase activity in treated onion plants with different bacterial bioagents, under artificial inoculation conditions
Column with a different letter significantly differ at P 0.05 according to Duncan's multiple range test

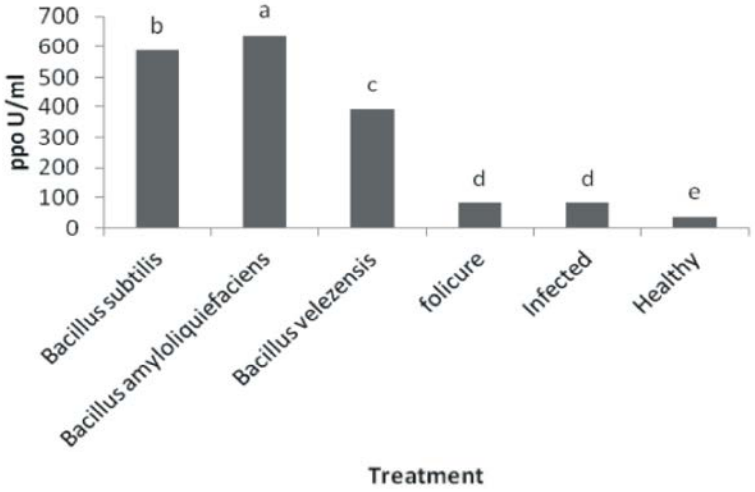

Fig. 6: Polyphenol oxidase (PPO) activity in treated onion plants with different bacterial bioagents, under artificial inoculation conditions
Column with a different letter significantly differ at P 0.05 according to Duncan's multiple range tests

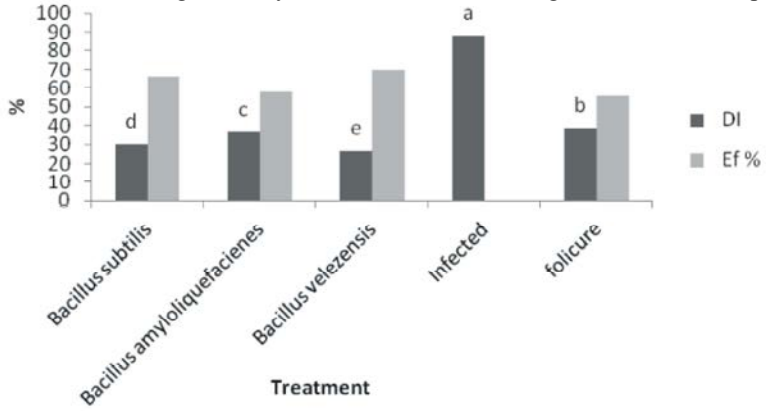

Fig. 7: Disease index (DI) and % efficiency of whit rot of onion plants treated with different bioagents,
Column with a different letter significantly differ at P 0.05 according to Duncan's multiple range test

DISCUSSION

White rot disease caused by *Sclerotium cepivorum* considered the most severe disease of onions (*Allium cepa*). *S. cepivorum* sclerotia give mycelium as a response to the presence of *Allium* root exudates [28]. Sixty eight bacterial isolates were examined for their antagonistic activities versus *Sclerotium cepivorum*, *in vitro*. Three antagonistic isolates (B4, B18 and B29) were gave higher inhibition against the pathogen. These isolates were selected and identified using conventional methods and 16SrRNA gene sequencing. They were identified as *B. subtilis, B. amyloliquefacienes* and *B. velezensis* respectively. Species related to group of *Bacillus* are known as well producers of variable compounds with different structures that have antagonistic effects for many pathogens. Most bioactive compounds produced by *Bacillus* are bacteriocins, polyketide, lipopeptides and siderophores. Generally, they have a wide range of antagonistic activity against different plant pathogenic fungi, bacteria and viruses [29]. *Bacillus* isolates under investigation were examined for their production of indoles and phosphate solubilization. Data revealed that the three bioagents have the ability to solubilize phosphate and have the capability to produce IAA. This result is in accordance with Singh *et al*. [30] who found that *Bacillus amyloliquefaciens* strain DSBA-11 had high ability to solubilize phosphorus (42.6 µg/ml) and produce IAA (95.4 µg/ml). The abilities of three *Bacillus* isolates to produce hydrogen cyanide, siderophores, protease, amylase and gelatinase were tested. Data showed that all selected isolates could produce siderophores, none of them could produce HCN but *B. amyloluqiefacenes* and *B. velezensis* showed good capability to produce amylase, protease and gelatinase enzyme. Similar results were obtained by Fan *et al*. Kaki *et al*. and Wulff *et al*. [31-33]. In general, *Bacillus* spp. has the ability to act as antibiosis depending on their capability to produce hydrolytic enzymes that degrades polysaccharides and nucleic acids of the pathogenic fungi [34]. Arrebola *et al.* [35] and Gao *et al.* [36] reported that *B. amyloliquefacienes* PPCB004 can produce acetoin as a major volatile compound while *B. velezensis* ZSY-1 strain could produce various antifungal and volatile compounds like 2-tridecanone, phenol (4- chloro-3-methyl) and pyrazine (2, 5- dimethyl) benzothiazole. Multiple mechanisms seem to be used by *Bacillus* spp. in the biocontrol of *S. cepivorum* such as: (i) activate the defense mechanisms of plant (ii) competition for iron through production of siderophores (iii) competition to establish an ecological site and metabolize root exudates (iiii) degradation of pathogenicity factors such as toxins [37, 38]. Activities of polyphenol oxidase and peroxidase had remarkable increasing in treated plants with bacterial bioagents. These enzymes are well associated with the defensive system of host plants. Similar study on tea blister blight disease showed greater enzymes production in plants that treated with PGPR than in control [39]. The results of this study developed effective and safe controlling for onion whit rot of disease by using bacterial bioagents.

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