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Biological Control of Potato Brown Leaf Spot Disease Caused by *Alternaria alternata* Using *Brevibacillus formosus* Strain DSM 9885 and *Brevibacillus brevis* Strain NBRC 15304

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Abstract

Brown leaf spot is one of the prevalent diseases caused by *Alternaria alternata* in different growing areas of potato worldwide. Eight *A. alternata* isolates were screened from forty-two isolates collected from different potato growing regions in four Egyptian governorates *viz*, North Sinai (Baloza), Beheira (El-Nubaria and Wadi El-Natrun), Ismailia (Abu Suweir, Fayed and Tell El-Kebir), Sharqia (New Salheya and El-Husseiniya). The virulence of the isolates was tested based on the Per cent of Disease Index (PDI) which ranged from 28.2% to 70.3% PDI by Alternaria isolates of Baloza and Fayed respectively. Two bacterial strains "*Brevibacillus formosus* strain DSM 9885, and *Brevibacillus brevis* strain NBRC 15304 were selected to control of *A. alternata*. The bacterial strains have a higher inhibitory effect on mycelial development and spore germination of *A. alternata*. To determine the effects of the bacterial strains on disease index and severity, the most virulent of *A. alternata* isolates were selected for greenhouse experiments where the potato plants were sprayed with bacterial strains individually and mixture treatments. Superior effect of treatments in disease reduction was observed when the two bacterial strains were combined. The effect of leaf age was studied where the leaf position has significant effect on disease progress. The changes of soluble protein in potato leaves due to *Brevibacillus* strains application were studied. Protein profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the plant treated with mixture of biocontrol agents able to synthesize some new proteins with maximum number of bands followed by treatment by *B. formosus* strain. The presence or absence of the bands in protein profiling might be responsible for resistance response against *A. alternata* in potato. The present work suggests that use of *B. formosus* strain DSM 9885, and *B. brevis* strain NBRC 15304 could be considered as potential management tools for reducing the impact of *A. alternata* causing brown leaf spot disease on potato.

Keywords: Biocontrol agents; Spore germination; Antagonism; Biochemical; Protein profiling

Introduction

Potato (*Solanum tuberosum* L.) is one of the major agricultural crops worldwide and plays an important role in Egyptian agriculture. In 2014 approximately 381.7 million tons of potatoes were produced worldwide. In Egypt, potato has an important position among all vegetable crops where potato production in 2014 exceeded 4.6 million tons, produced on approximately 172.000 ha [1]. Potato crop is vulnerable to infect by several pathogenic fungi. Along with the devastating potato diseases brown spot is caused by *A. alternata* (Fries) Keissler. It is distributed over a wide range of climatic conditions so it can be found in many potatoes growing regions of the world [2]. *Alternaria* diseases on potato cause yield losses and reduce the quality of the crop and very difficult to control [3]. *A. alternata* is one of the prevalent pathogens causing potato brown leaf spot in different parts worldwide [4]. *A. alternata* mainly affects the potato leaves and leads to brown leaf spots. This disease causes a risk to crop production and significant yield losses especially in case of severe infection where losses result from reduced photosynthetic area, loss of weakened leaves plant and increases its susceptibility to infection, subsequently increases the imbalance between nutrient demand in the tubers and nutrient supply from the leaves, subsequently leading to reduced yields [5,6]. The quality and quantity of potato yield may be reduced by infections caused by pathogens that attack both the aboveground parts of potato plants [3,7,8]. Crop losses due to *A. alternata* are around 20 percent; but there have been cases of 70% to 80% losses in case of severe infections or when the disease is combined with other disease such as early blight [9,10]. Also, there is no major resistance gene for *A. alternata* is known. Genetic sources for partial resistance have been determined within some potato wild species [11,12]. So, this disease is one of the destructive diseases in most potato growing areas [13]. To suppress *Alternaria* spot disease causal agents and to prevent the losses it causes, potato fields are intensively sprayed with fungicides [14,15]. Fungicides of various chemical groups are currently used worldwide to control *Alternaria* spp. on potato. Optimization of fungicide use for the control of *Alternaria* diseases is still a considerable challenge due to the capacity of pathogen to produce huge amounts of inoculum [15], so there is a challenge of selecting fungicide resistance in target populations of *Alternaria* spp. [16]. The high efficiency of these chemical pesticides can result in environmental contamination and the effect of pesticide residues on food, in addition to social and economic impacts. Several investigations have been carried out to improve *Alternaria* disease management and to reduce the number of sprays [17]. Eco-friendly methods using biocontrol agents and induce resistance agents to suppress plant disease provides a useful alternative tool to use these evaluated agents with similar targets [3]. Biological control and use of antagonistic microorganisms such as bacteria has considered as a promising alternative strategy. Indeed, these bio-pesticides provide many advantages in term of ecofriendly disease control methods. Antagonistic bacterial isolates are widely

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used for the biocontrol of fungal plant diseases [18]. Rhizo-Bacteria are one of the important groups of biological control agents which have revolutionized the field of biological control of several plant pathogens [19-22]. They play important role in induced systemic resistance due to physical and mechanical strength of cell wall and effects on biochemical and physiological reactions of the host plant through synthesis of chemical defense against fungal pathogens [23-26]. The genus Bacillus is distributed widely in environment and includes thermophilic, alkalophilic, and halophilic bacteria that utilize several sources of carbon for heterotrophic and autotrophic growth. Bacillus is one of the most common genera of gram-positive bacteria, isolated from several environmental habitats [27]. *B. formosus* and *B. brevis* are important species according to gene sequence study by Shida et al. [28] where some strains of them were studied for their activities as biocontrol agents against several plant pathogens due to their antibacterial and antifungal effects [29,30]. According to genome sequencing studies for taxonomy of genes and phylogenomics of Bacillus-like bacteria the *B. formosus* DSM 9885 was deposited in seven culture collections [31]. Some of these strains have biocontrol potential against different phytopathogenic fungi and can produce a hyperthermostable chitinase [32]. Also, several strains of *B. brevis* were studied as biocontrol agents for controlling a wide range of plant pathogens [33], different strains also evaluated and encouraged as potential plant growth for enhancing the growth and crop productivity [30,34,35]. Several studies recently reported that the use of *Brevibacillus* strains as biocontrol agents could reduce amounts of chemical fungicides applied for control of phytopathogenic fungi [20,31,33,34].

The aim of this work was to study the efficacy of two bacterial strains *B. formosus* "strain DSM 9885, and *B. brevis* "strain NBRC 15304" as control agents of *A. alternata* to reduce fungicide applications in brown leave spot diseases management. The changes of soluble protein in potato leaves due to Brevibacillus strains application were studied through protein profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Materials and Methods

Survey, collection and identification of the pathogens

Potato leaves showing typical brown spot symptoms were collected from different growing areas in four Egyptian Governorates viz, North Sinai (Baloza), Beheira (El-Nubaria; Wadi El Natrun); Ismailia (Abu Suweir, Fayed and Tell El Kebir); Sharqia (New Salheya; El Husseiniya) during 2015-2016 to identify the variability of the pathogen and their ability to control by biological agents. The most aggressive isolates were selected to other *in vitro* and greenhouse experiments. The pathogens were identified based on their cultural and morphological characters.

Fungal cultures and inoculation

A single spore isolate of *A. alternata* which caused symptoms in potato leaf tissue was used. Conidia were maintained on filter paper at 4°C. Pure cultures of the isolate were produced by placing a small section of filter paper containing conidia on Potato Dextrose Agar (PDA). For inoculums production, cultures of *A. alternata* were cultured on Potato Dextrose Agar (PDA) at 18°C for 14 days. Conidia were collected by flooding the surface of the Petri dish with 5 ml sterile distilled water, and gently scraping the surface of the media with an L-shaped glass rod to collect the conidia. Then the conidial suspension was stirred with a magnetic stirrer for 1 h and strained through cheesecloth to exclude the mycelial fragments. The concentration was then adjusted to 1×10^5 conidia/ml using a hemocytometer, the fungal purification and inoculum were prepared according to Soleimani and Kirk [9].

Pathogenicity test and virulence of *Alternaria alternata* isolates

The pathogenicity of purified *A. alternata* isolates was tested and proved by Koch's Postulates. Potatoes were planted in each pot with three replicates under greenhouse conditions. The conidial suspension from two weeks old culture of *A. alternata* was used. The conidial concentration adjusted to (5×10^5 spores ml^{-1}) using haemocytometer. Spore suspensions were sprayed on the plants of 30-day-old plants. The plants sprayed with sterile water served as control. Inoculated plants were covered tightly with plastic sheet, after 24 hours the cover was removed and the humidity was maintained by spraying tap water. The plants were grown in a greenhouse for the symptoms appeared and developed. The severe symptoms were observed on 12 to 15 days after inoculation and the disease intensity was recorded. The symptoms were observed and compared with the original symptoms. The fungal isolates were reisolated from artificially inoculated potato leaves and compared with original culture isolates and they were the same. The pathogenicity test was carried out according to Stammler [36]. The disease index was calculated using nine grade scale from 0-9 where, where 0=no spots and 9=brown spots visible more than 60% as leaf area spotted. The Per cent Disease Index (PDI) was calculated by using formula of McKinney [37]:

$$PDI = \frac{Over\ all\ of\ numerical\ rating}{Total\ number\ of\ leaves\ observed} \times \frac{100}{Maximum\ disease\ grade}$$

Morphological and molecular identification of biocontrol agents

The bacterial isolates used in this study were kindly provided by Soil Fertility and Microbiology Department, Desert Research Center, which evaluated against different fungal pathogens, and being obtained in previous investigation [20]. Selected isolates used in present study were identified to molecular level using partial 16S rRNA gene sequence technique based on Berg [38]. In Sigma Scientific Services Co., bacterial 16S rRNA gene sequences were amplified by PCR using the eubacterial primer pair 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3') [39]. The PCR product was sequenced with Genetic Analyzer sequencer, Data Collection v3.0, Sequencing Analysis v5.2 (Foster City, USA). Obtained sequences were aligned with reference RNA sequences from National Center for Biotechnology Information (NCBI) data base [20].

Bacterial inoculum preparation

Bacterial strains were maintained in 80% glycerol (v/v) at -80°C as stock cultures. In order to culture process, a loopful of inoculum was streaked on nutrient medium (NA) plates. Each strain was evaluated to control of *A. alternata* isolates. To harvest the metabolites, fresh cells were obtained from stock cultures and grown in nutrient broth medium at room temperature. 100 ml of nutrient broth was inoculated and incubated for 48 h at room temperature in a rotary shaker (80 round/min). The bacterial culture was centrifuged 10000 rpm for 10 min, and the supernatant was discarded. The cell pellets were suspended in sterile 0.85% NaCl then centrifuged again under the same conditions. The supernatant was discarded and washed bacterial cells were re-suspended in sterile distilled water. The concentration of cells in the suspension was spectrophotometrically adjusted to 108 CFU/ml and used for greenhouse pot experiments [40].

Antagonistic effect of bacterial isolates against *A. alternata*

The antagonistic activity of two bacterial strains was investigated against *A. alternata* by dual culture technique [41, 42] using PDA medium. For testing the antagonistic effect of bacterial strains, each of them was streaked in center of sterile petri dish on potato dextrose agar

(PDA). One disc (0.5 cm in diameter) of *A. alternata* was placed on the side of the same petri dish at 10 mm distance. Petri dishes with fungal cultures and free of bacteria were used as control. Each treatment was carried out with four plates per replicate. Periodical observations on the ability of bacterial strains to colonize the pathogen were calculated as percent inhibition of mycelial growth of pathogen by using the following formula [43].

$$\text{Percent Inhibition}\left(\text{PI}\right)=\frac{C-T}{C}\times 100$$

Where, T: Growth of pathogen in dual culture plates and C: control plates

Effects of biocontrol agents on spore germination of *A. alternata*

Inhibition of spore germination *in vitro*: The effect of bacterial strains on spore germination was studied *in vitro* according to the method of Nair and Ellingboe [44]. For the test, concentration of bacterial suspension (10^8 cells mL^{-1}) was prepared. A drop of bacterial cells was deposited on dried clean glass slides as a film. A drop of the spore suspension of the pathogen was spread over this film. Control treatment was prepared as a film of sterilized distilled water. Percentage of spore germination was determined microscopically using 400 folds magnification [45]. Percentage of germination was obtained using the following formula [46]

$$\text{Percentage of germination}=\frac{\text{Number of germinated spores}}{\text{Total number of spores}}\times 100$$

Inhibition of spore germination on detached leaflets: Effects of a separate and combined application of the tested bacterial strains were studied against spore germination of *A. alternata* on detached potato leaflets. Suspension of each bacterial strain and their mixture were sprayed on potato leaflets by using of an atomizer sprayer. Directly after spraying, drops (20 µl each) containing spore suspension of *A. alternata* were placed on the leaflets. Then the leaflets were put in plastic boxes with humid filter paper and covered to maintain high relative humidity. The boxes were placed in incubators. After one day of incubation, germination of *A. alternata* spores was determined where the leaflet bearing a drop of the interacting microorganisms were placed on glass slides and incubated for 2 h at room temperature (22°C), and examined microscopically, germination was determined in samples of 50 conidia of *A. alternata* for each treatment that were examined in each of 5 drops from three different leaflet replicates [47].

Greenhouse experiment

The experiment was conducted as a randomized complete block design with three replicates (ten plants/replicate) for each treatment. Potato tubers of Spunta cultivar were used. Potato seed tubers were planted in plastic pots (50 cm diameter) containing sandy loam soil. The treatments were added as single and mixture of two bacterial strains (*B. formosus* strain DSM 9885, and *B. brevis* strain NBRC 15304. Through this experiment the disease severity and disease index were calculated on plant under greenhouse conditions. Two days after foliar application of biocontrol agents as well as control treatment by water, the percentage of foliage protection against *A. alternata* was evaluated by using the detached leaf techniques where four leaves per each of the three different leaf positions (top, middle and lower) part were detached from ten plants per treatment and replication then transferred to the laboratory. The detached leaves were artificially inoculated with *A. Alternata* by placing a 50 µl droplet of conidial suspension (1×10^5 conidia/ml) on the center of the leaflet and incubated with humid filter paper in a growth chamber in darkness at 18°C for one day, then the treated leaflets were incubated continually in a growth chamber at 21°C and fluorescent tube light for 16 h day. Disease symptoms development we observed daily from the third to seventh day after inoculation by visual assessment of the leaf area showing brown leaf spot. Also, Disease incidence and severity were calculated based on percentage of damaged potato leaf area and affected number of plants under greenhouse conditions. Disease severity was recorded by estimating the lesions on a scale from 1 to 7, where: 1=no lesions, 2=a few circles, 3=up to 30%, 4=31% to 40%, 5=41% to 50%, 6=51% to 60%, 7=61% to 100%, (most severe symptoms) of leaf area with brown leaf spot symptoms. Then the following formula was applied:

$$DS=\frac{\sum\left(n\times c\right)}{N}$$

Where, DS=disease severity, n=number of infected plants per category, c=category number and N=total number of examined plants.

Protein profiling and gel preparation

Protein of potato leaves extracted from treated plants by pathogen and biocontrol agents, the samples washed several times with distilled water and blotter dried before protein extraction. Amount of 1.0 g of each sample was grinded by mortar using 1:5 leaves: extraction buffer. The suspension was centrifuged at 10000 rpm for 30 min at 4°C. The supernatant was collected and used for profiling of protein [48]. SDS-PAGE was done to get banding pattern of soluble protein. Soluble protein was electrophoresed by 12% SDS polyacrylamide gel, based on the method of Laemmli [49]. Stacking, resolving gel and sample loading were prepared according to Rajik et al. [50].

Statistical analysis

The data obtained was subjected to analysis of variance technique using completely randomized design (CRD) following Gomez and Gomez [51].

Results and Discussion

In this study, laboratory and greenhouse experiments were performed to determine the effects of two bacterial strains *B. formosus* strain DSM 9885, and *B. brevis* strain NBRC 15304 which designated in this paper (*Brf1 and Brb2*) respectively, as potential biocontrol agents against screened eight *A. alternata* isolates designated (*Alt1–Alt8*).

Isolates of *A. alternata* and their virulence

Potato leaves showing typical brown leaf spot symptoms were collected from some potato growing areas in four Egyptian governorates viz., North Sinai, Beheira, Ismailia, Sharqia during 2015-2016. Forty-two isolates were obtained from infected potato plants. The isolates were grown on PDA and screened based on variations in culture morphology then preliminary test of pathogenicity (data not shown). Eight *A. alternata* isolates were selected for experiments of the present study. The tested isolates were isolated from eight different locations Baloza (North Sinai), El-Nubaria; Wadi El Natrun (Beheira); Abu Suweir, Fayed and Tell El Kebir (Ismailia); New Salheya; El Husseiniya (Sharqia) to assess their pathogenicity and their ability to controlled by tested bacterial strains as potential biocontrol agents. Isolates of *A. alternata* varied in pathogenicity on potato (Table 1). The most virulent isolate was *Alt5* (70.3% PDI), followed by *Alt2* (65.5% PDI). While *Alt1* was the least pathogenic. These results are agreed with some previous studies on pathogenicity of *A. alternata* proved

Sr. No.	Isolates	Isolation place	PDI %*
1	*Alt1*	North Sinai (Baloza)	28.33e
2	*Alt2*	Beheira (El-Nubaria)	63.66b
3	*Alt3*	Beheira (Wadi El Natrun)	38.5d
4	*Alt4*	Ismailia (Abu Suweir)	61.67b
5	*Alt5*	Ismailia (Fayed)	71.33a
6	*Alt6*	Ismailia (Tell El Kebir)	30.66de
7	*Alt7*	Sharqia (New Salheya)	42.67cd
8	*Alt8*	Sharqia (El Husseiniya)	46c
* Means with the same letter are not significantly different.			

Table 1: Sources and virulence of different *A. alternata* isolates.

Treatments	Fungal Isolate															
	Alt1		*Alt2*		*Alt3*		*Alt4*		*Alt5*		*Alt6*		*Alt7*		*Alt8*	
	Growth	*Reduction %*	*Growth*	*Reduction %*	*Growth*	*Reduction %*	*Growth*	*Reduction %*	*Growth*	*Reduction %*	*Growth*	*Reduction %*	*Growth*	*Reduction %*	*Growth*	*Reduction %*
Brf1	2.50	70.9	4.60	51.1	3.40	64.2	4.20	54.3	3.60	62.1	2.80	68.2	2.20	76.8	1.93	79.7
Brb2	5.90	31.4	6.20	34	4.57	51.9	8.20	10.9	7.40	22.1	5.50	37.5	4.80	49.5	4.57	51.9
Brf1+Brb2	2.23	74.1	2.50	73.4	2.23	76.5	3.30	64.1	2.93	69.2	2.60	70.5	1.90	80	2.17	77.2
Cont.	8.60	0	9.40	0	9.50	0	9.20	0	9.50	0	8.80	0	9.50	0	9.50	0
$LSD_{0.05}$	*0.25*	-	*0.46*	-	*0.42*	-	*0.44*	-	*0.76*	-	*0.46*	-	*0.39*	-	*0.41*	-

Table 2: Effect of *Brevibacillus formosus* strain DSM 9885 (*Brf1*), and *Brevibacillus brevis* strain NBRC 15304 (Brb2) and their mixture (*Brf1+Brb2*) on mycelial growth of *A. alternata* isolates (*Alt1-Alt8*).

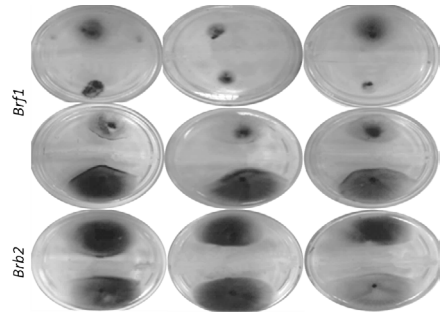

Figure 1: Effect of biocontrol agents (*Brf1*and *Brb2*) on the mycelial growth of various tested pathogenic isolates.

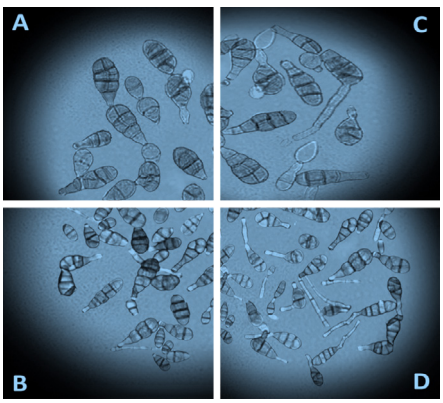

Figure 2: Germination of two isolates of *Alternaria alternata* spores. A: treated by *Brf1*; B: treated by *Brb2;* C, D: control.

under semi controlled condition where the variation of pathogenicity was reported with typical brown spot symptoms when observed on all the inoculated plants of several crops such as apple [52]. In present study, the maximum pathogenicity caused by *Alt2* and *Alt5* may be due to more toxin production as well as their adaptability to favorable environment conditions [53,54].

Effect of biocontrol agents on linear growth of *Alternaria alternata*

The antagonistic effect of tested bacteria against *A. alternata* was studied. *B. formosus* "strain DSM 9885, and *B. brevis* "strain NBRC 15304" were used *in vitro* to evaluated their effects on mycelial growth of *A. alternata* isolates using dual culture technique. PDA medium without adding the bacteria served as control. The mycelial growth reduction of fungi was calculated according to of the inhibition zones as a distance between fungal growth and bacterial colony [42]. The interactions on solid medium revealed the antagonistic effect of the bacterial strains used throughout this study. In dual cultures with evaluated bacterial strains, a more evident inhibitory action (clear zone of mycelial inhibition) was observed. The highest inhibitory effect was recorded in the six or seven days of cultivation with *A. alternata* isolates. The highest inhibition effect was detected with mixture of *Brf1* and *Brb2*, while the *Brb2* as a single treatment presented the least effect against all tested *A. alternata* isolates. The growth reduction rates were ranged from 51% to 79.7% in *Alternaria* isolates treated by *Brf1*, while the fungal growth was reduced by 10% to 51.9% with Brb2 compared to the mixture treatments where the fungal growth was reduced from 64.1% in *Alt4* to 80% in *Alt7* (Table 2). In the case of *Brf1,* the inhibition zone was clearer than the other that appears in the presence of *Brb2*, this aspect being indicated to more stable and a higher inhibitory activity. However, in early stage of the mycelium development, both of tested strains inhibited of mycelium extension and restricted the growth of fungal (Figure 1). It seems that *Brevibacillus* strains excreted metabolites that act as a barrier between the fungi and bacteria, the mycelium development being restricted due to the synthesis of compounds with antifungal activity surrounding colonies, at the same time, the mixture of two isolates can inhibit the fungal growth with higher effect than single treatment by each other, that may be due to the excretion of lytic enzymes or other compounds with fungicidal activity [27,29,55].

Effects of biocontrol agents on spore germination of *A. alternata*

Spore germination is one of most principal factors of survival,

dispersal and virulence of pathogenic fungi. Effects of the *Brevibacillus* strains on spore germination of *A. alternata* ware tested through slide test *in vitro* and detached leave test. Most of *A. alternata* spores germinated in control samples were ranged from 64% to 86% germination in slide test, and 52% to 78% on detached leaves (Tables 3 and 4). Regarding to the effect of treatments in slide test, the germination spore rates were 25%, 30%, 33% in *Alt2, Alt4, Alt5* in case of *Brf1* treatment, and 28%, 51%, 40% by *Brb2*, compared to control (84%, 64%, 86%) germination spores of *Alt2, Alt4 and Alt5* (Table 3 and Figures 2A-2D). In detached leave test, the obtained results were in line with slide test. The means of spore germination of *A. alternata* were ranged from 25% to 54.6% in case of treatments by *Brf1*, while the germination rates were found from 34% to 66% resulted by *Brb2*. In combined treatment, the germination spore rates were ranged from 20% to 47% compared to control (52% to 78%). Significant differences were observed between effects of each bacterial isolate individually and mixed on inhibition of spore germination compared to mixture treatment (Table 4). Previous experimental results indicated that *Bacillus sp.* produce antibiotics such as *bacilysin, iturin, mycosubtilin* and siderophores which are responsible for the inhibition of fungal spore germination [56-58].

Assessment of *Brevibacillus* strains on brown leaf spot disease of potato

Disease suppression in the detached leaves which treated in greenhouse: This experiment was designed to test the hypothesis that disease progress is affected by the leaf age or leaf position in the lower, middle and upper parts of the leaf position. In terms of disease suppression, all of the treatments were effective compared to the untreated, inoculated control (Table 5). The results obtained from the detached leaves experiment showed significant difference regarding disease index reduction among biological control agents of *Brevibacillus*. However, it was clear that in both treated potato plants, application of the mixture of *Brf1* and *Brb2* was more encouraging to enhancement of disease resistance compared to the separate treatment. These two *Brevibacillus* strains have indicated higher reduction of disease index in treated detached leaves where High reduction was obtained by *Brf1+Brb2* as treatments combined on upper leaves against pathogenic isolates *Alt2* followed by *Alt5* where the mean of disease index recorded (1.49 and 1.7) respectively. *Brf1* and *Brb2* or their mixture showed significant reduction in disease index on detached leaves. Also, the observed symptoms indicated that leaf position has

Treatments	Fungal isolate							
	Alt1	*Alt2*	*Alt3*	*Alt4*	*Alt5*	*Alt6*	*Alt7*	*Alt8*
Brf1	20.00	25.00	16.00	30.00	33.00	21.33	22.00	30.00
Brb2	35.00	28.00	20.00	51.00	40.00	33.00	30.00	38.00
Cont.	78.00	84.00	72.00	64.00	86.67	68.00	82.00	76.00
$LSD_{0.05}$	6.21	4.76	4.00	7.74	4.80	6.86	4.76	8.63

Table 3: Effect of *Brevibacillus formosus* strain DSM 9885 (*Brf1*), and *Brevibacillus brevis* strain NBRC 15304 (*Brb2*) on spore germination of *A. alternata in vitro*.

Treatments	Fungal isolate							
	Alt1	*Alt2*	*Alt3*	*Alt4*	*Alt5*	*Alt6*	*Alt7*	*Alt8*
Brf1	43.00	25.33	31.00	53.00	54.67	40.00	38.00	42.00
Brb2	62.67	37.00	34.00	66.00	61.00	48.00	47.00	51.00
Brf1+Brb2	35.00	20.00	24.67	45.00	47.00	33.00	30.00	38.00
Cont.	52.00	55.33	64.00	75.00	78.00	64.00	78.00	61.00
$LSD_{0.05}$	8.17	6.50	6.41	5.41	3.69	4.80	4.31	3.39

Table 4: Effect of *Brevibacillus formosus* strain DSM 9885 (*Brf1*), and *Brevibacillus brevis* strain NBRC 15304 (*Brb2*) on spore germination of *A. alternata* on detached leaves.

Fungal isolate	Treatments	Age	*Mean of disease index
Alt2	*Brf1*	Upper	2.23 nop
		Middle	3.70 jk
		Lower	6.22 de
	Brb2	Upper	1.82 qr
		Middle	4.20 hi
		Lower	6.33 de
	Brf1+Brb2	Upper	1.49 r
		Middle	2.80 lm
		Lower	4.35 h
	Cont.	Upper	3.50 k
		Middle	5.77 f
		Lower	9.73 a
Alt4	*Brf1*	Upper	2.53 mno
		Middle	4.30 h
		Lower	6.60 d
	Brb2	Upper	2.40 no
		Middle	4.93 g
		Lower	7.10 c
	Brf1+Brb2	Upper	1.90 pq
		Middle	3.10 l
		Lower	5.20 g
	Cont.	Upper	3.90 ij
		Middle	6.60 d
		Lower	9.87a
Alt5	*Brf1*	Upper	2.20 op
		Middle	4.00 hij
		Lower	6.00 ef
	Brb2	Upper	2.60 mn
		Middle	4.20 hi
		Lower	6.50 d
	Brf1+Brb2	Upper	1.70 qr
		Middle	3.00 l
		Lower	5.10 g
	Cont.	Upper	3.00 l
		Middle	5.00 g
		Lower	8.80 b

* Means with the same letter are not significantly different.

Table 5: Effect of biocontrol agents on potato brown spot disease index. Pre-treated, detached potato leaves from different positions on plants were artificially inoculated *in vitro* with 50 µl suspensions containing 5×10^5 spore/ml of *A. alternata*.

a significant effect on the lesion growth rate of *A. alternata* on leaves from upper part of the plant. However, disease index was significantly greater on untreated plots. Disease progress was showed on leaves from three parts of the plant. A disease symptom developing was low at the apex, moderate in the middle, and high in the lower part of the plants in both treatments. This is in agreement with assessments by Visker [59], Soleimani and Kirk [9]. They have found that older leaves in the lower part of the plant seemed to be more susceptible to brown leaf spot disease than younger leaves in the upper part. So, they reported that leaf position is a significant factor in potato resistance. This may be due to induce the systemic acquired resistance as a result of treatment with biocontrol agents, and their ability to stimulate the plant resistance in younger leaves faster than the older leaves [9,27,60]. There are several reports on the reliability of the detached leaflet method as a screening technique and its correlation with laboratory and field or greenhouse disease data [59,61,62]. The detached leaflet screening method indicated that this technique provides a reasonable assessment of brown leaf spot resistance, and could be a reliable screening system [9].

Assessment of *Brevibacillus* strains effects on brown leaf spot disease of potato under greenhouse conditions

Greenhouse experiment was carried out to evaluate the antifungal activity of two *Brevibacillus* strains against *A. alternata*. According to data obtained from *in vitro* experiments, the most pathogenic *Alternaria* isolates (*Alt2, Alt4* and *Alt5*) were selected and tested under greenhouse conditions. Three treatments were compared: *Brevibacillus*1 (*Brf1*), *Brevibacillus* (*Brb2*), mixture of both strains (*Brf1+Brb2*), and water spray as control treatment. The two *Brevibacillus* starins were effective to disease index and severity of brown leaf spot symptoms. This observed reduction was different due to treatments, Superior disease reduction effect was observed when the two bacterial strains were combined. The most effective strain was *Brf1* that reduced the disease incidence by 58.3%, 54.5% and 66%, respectively in case of *Alt2, Alt4* and *Alt5* respectively. Superior effect of treatments in disease reduction was observed when they were combined. The highest record of disease reduction in order of 62.5% and 71.7% were obtained for the applied combined treatment against *Alt2* and *Alt5* isolate (Table 6). Similar trend was recorded concerning the severity of brown leaf spot disease. Most of potato plants receiving *Brevibacillus* treatments have significant reduction in disease severity with combined of both two strains application. High reduction as 37% and 50% were obtained from treatments by *Brf1* against *Alt2* and *Alt4* and reached up to 58% when both bacterial strains combined. Individual application *Brf1* and *Brb2* or their mixture showed significant reduction in disease incidence as well as severity. The results also showed that there was more disease development in case of treatment by *Brb2*, than at the *Brf1*. However, it was clear that, application of tested *Brevibacillus* strains as mixture was the most effective of all of the treatments against tested pathogenic isolates for enhancing disease resistance. Similar results were obtained on two different potato cultivars against different fungal pathogens [9,21]. Application of *Brevibacillus* strains has indicated higher resistance of plants against tested *Alternaria* isolates on the potato. However, the greenhouse data has indicated that the potato plants which treated with combined treatment were much healthier than for the other treatments. Previously some studies reported similar effects with the application of Bacillus strains against different pathogenic fungi on sage plants and potato, respectively [20-22]. In present study, the results showed that these two *Brevibacillus* strains are able to decrease *A. alternata* disease infection when given as a foliage spray. The encouraging effect and performance of tested biocontrol agents on disease severity and incidence on potato plants and pathogen growth as well as spore germination might be due to the ability of *Brevibacillus* to suppress the fungal pathogen. Also, these effects may be associated with the activation of some novel defense pathways. Some previous studies on genomic sequences of different *Brevibacillus* strains mentioned to their responsibility for synthesis of antifungal compounds [28,29,57]. The systemic resistance also has been reported in other plant path systems, against leaf disease [63-66].

Fungal isolate	Treatment	DI	Reduction %	DS	Reduction %
Alt2	*Brf1*	*20.0 mno	58.3	36.0 fg	37.9
	Brb2	28.0 ijk	41.7	42.0 e	27.6
	Brf1+Brb2	19.3 no	62.5	28.0 ijk	51.7
	Cont.	48.0 d	0	58.0 abc	0
Alt4	*Brf1*	25.0 klm	54.5	30.0 hijk	50.8
	Brb2	31.0 ghij	43.6	34.0 fgh	31.7
	Brf1+Brb2	21.0 lmn	61.8	26.0 jkl	58.7
	Cont.	55.0 bc	0	63.0 a	0
Alt5	*Brf1*	18.0 no	66.0	33.0 fghi	45.0
	Brb2	21.0 lmn	60.4	37.0 f	38.3
	Brf1+Brb2	15.0 o	71.7	29.0 hijk	51.7
	Cont.	53.0 c	0	60.0 ab	0

* Means with the same letter are not significantly different.

Table 6: Effect of biocontrol agents on potato brown spot disease index (DI) and severity (DS) under greenhouse conditions.

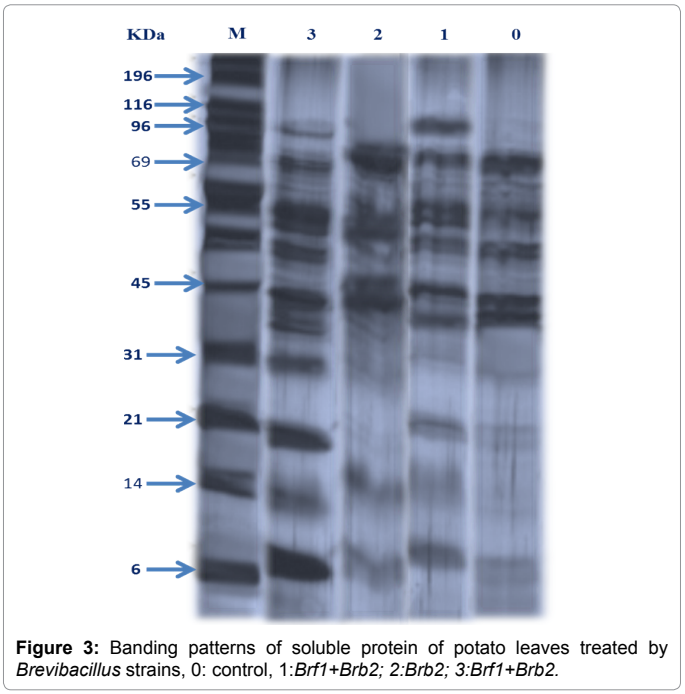

Figure 3: Banding patterns of soluble protein of potato leaves treated by *Brevibacillus* strains, 0: control, 1:*Brf1+Brb2; 2:Brb2; 3:Brf1+Brb2.*

Protein profiling

Leaf protein contents of control, *Alternaria* infected, and *Brevibacillus* treated leaves are shown in Figure 3. In response to biocontrol agent's inoculation, the soluble protein contents increased significantly in comparison to control. SDS-PAGE is used for finding the banding pattern of proteins. Protein profiling was done to determine whether some new protein was associated with treatment and resistant to *A. alternata* in potato cultivar (Spunta) or not. The banding patterns of protein of different treatments were (14,16,19) bands in treatments with *Brf1, Brb2 and Brf1+Brb2* respectively compared to control (11) bands. The highest number of bands was found in treated potato leaves by mixture of two tested *Brevibacillus* strains, and minimum number of bands was in control plant. The banding pattern of proteins from figure represented that some proteins of different molecular weight was found in treated plant which was not found in control. Similarly, some new bands were also found in mixture treatment not found in treatment by bacteria individually. The presence or absence of protein bands might be due to the inducing effects of *Brevibacillus* strains in plant which may also be responsible factors for enhancing of potato defense mechanism against *A. alternata*. Similar results were obtained by Biswas [67] reported that some new proteins were associated with resistance to *Bipolaris sorokiniana* induced by crude extracts of *Chaetomium globosum*. Also, Rajik et al. [50] and Romeiro [68] reported that protein profiling by SDS-PAGE revealed that some new protein is synthesized due to application by some induce resistance agents against *F. oxysporum f. sp. lycopersici* in tomato. Subsequently, given the results obtained by Silva et al. [69] where they found increased activity of some enzymes in a tomato rhizobacteria interaction against the pathogen *Pseudomonas syringae*, which was interpreted to mean

that the rhizobacteria induced the systemic resistance in the tomato plants. However, the genus *Brevibacillus* includes a high diversity of thermophilic and halophilic strains which have ability to survive in harsh conditions and able to suppress wide range of plant pathogens. Therefore, some of *Brevibacillus* strains can be used as a source of many biotechnologically important enzymes such as a-amylase, xylanase and chitosanase [27,60], and can be play an effective role in control of some phytopathogenic fungi like *A. alternata* in potato plants.

Conclusion

In this study, the greenhouse and laboratory experiments have been performed to characterize the potential effect of two *Brevibacillus* strains against important fungal pathogen *A. alternata*. These experiments demonstrated that the use of the tested bacterial strains can enhance resistance to brown leaf spot in potato. The infection caused by *A. alternata* were observed in treated plants by biocontrol agents. Brown leaf spot severity was most significantly reduced by mixture of tested bacterial strains. The linear mycelial growth and spore germination of pathogenic fungi were inhibited by treatments which were confirmed by the results of *in vitro* and greenhouse experiments. Both of tested Brevibacillus strains reduced disease symptoms, and the effect was determined *in vitro* through detached leaves and under greenhouse conditions. Protein profiling by SDS-PAGE revealed that some bands of protein are produced due to application of biocontrol agents. The presence or absence of the bands in protein profiling might be responsible for induce resistance of potato plants against *A. alternata*. It may be concluded that, *B. formosus* strain DSM 9885, and *B. brevis* strain NBRC 15304 could be considered as part of management tools for reducing the impact of *A. alternata* causing brown leaf spot disease on potato.

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