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 (Balaza, Beheira (El-Nubarra and Wadi El-Natrun), Ismailia (Abu Suweir, Fayed and Tell El-Kebir), Sharqia (New Salhaya 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 *A. alternata* isolates of Balaza 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 [5,7,8]. 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]. Fungiocides 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 used, distribution, and reproduction in any medium, provided the original author and source are credited.

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used for the biocontrol of fungal plant diseases [18]. Rhizobacteria 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 an 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 hyperthermophile 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 spot disease 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 inoculations 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 × 10⁷ 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 × 10⁷ 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 Stammiller [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 McKinion [37]:

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

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 GGG 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) database [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].

\[
\text{Percent Inhibition (PI)} = \frac{C - T}{C} \times 100
\]

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 (10^5 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 was 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 x 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:

\[
DS = \frac{\sum (n \times c)}{N}
\]

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 blotted dried before protein extraction. Amount of 1.0 g of each sample was ground 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, Sharquia 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 Balboa (North Sinai), El-Nubaria; Wadi El Natrun (Beheira); Abu Suweir, Fayed and Tell El Kebir (Ismailia); New Salheya; El Husseiniya (Sharquia) 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...
A. alternata isolates (Alt1-Alt8).

Table 2: Effect of biocontrol agents on linear growth of *Alternaria alternata* isolates.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Alt1</th>
<th>Alt2</th>
<th>Alt3</th>
<th>Alt4</th>
<th>Alt5</th>
<th>Alt6</th>
<th>Alt7</th>
<th>Alt8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Reduction %</td>
<td>70.9</td>
<td>51.1</td>
<td>64.2</td>
<td>54.3</td>
<td>62.1</td>
<td>80.5</td>
<td>70.5</td>
<td>80.0</td>
</tr>
<tr>
<td>Growth Reduction %</td>
<td>59.1</td>
<td>34.0</td>
<td>42.0</td>
<td>30.0</td>
<td>42.0</td>
<td>35.0</td>
<td>35.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Cont.</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>0.25</td>
<td>0.42</td>
<td>0.42</td>
<td>0.44</td>
<td>0.46</td>
<td>0.46</td>
<td>0.46</td>
<td>0.41</td>
</tr>
</tbody>
</table>

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 evaluate 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 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 (Brf1+Brb2) on mycelial growth of *A. alternata* isolates (Alt1-Alt8).

### 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.](image1)

![Figure 2: Germination of two isolates of *Alternaria alternata* spores. A: treated by Brf1; B: treated by Brb2; C, D: control.](image2)

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].

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* were tested through slide test *in vitro* and detached leaf 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 leaf 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 the suppression of disease than younger leaves in the upper part. So, they reported 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].

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Treatments</th>
<th>Age</th>
<th><em>Mean of disease index</em></th>
</tr>
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<tbody>
<tr>
<td><strong>Alt2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brf1</em></td>
<td>Upper</td>
<td>2.23</td>
<td>nop</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>3.70</td>
<td>jk</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>6.22</td>
<td>de</td>
</tr>
<tr>
<td></td>
<td>Upper</td>
<td>1.82</td>
<td>qr</td>
</tr>
<tr>
<td><strong>Brb2</strong></td>
<td>Middle</td>
<td>4.20</td>
<td>hi</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>6.33</td>
<td>de</td>
</tr>
<tr>
<td><strong>Brf1+Brb2</strong></td>
<td>Middle</td>
<td>2.80</td>
<td>lm</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>4.35</td>
<td>h</td>
</tr>
<tr>
<td><strong>Cont.</strong></td>
<td>Upper</td>
<td>3.50</td>
<td>k</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>5.77</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>9.73</td>
<td>a</td>
</tr>
<tr>
<td><strong>Alt4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brf1</em></td>
<td>Upper</td>
<td>2.53</td>
<td>mno</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>4.30</td>
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<tr>
<td></td>
<td>Lower</td>
<td>6.60</td>
<td>d</td>
</tr>
<tr>
<td><strong>Brb2</strong></td>
<td>Upper</td>
<td>2.40</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>4.93</td>
<td>g</td>
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<tr>
<td></td>
<td>Lower</td>
<td>7.10</td>
<td>c</td>
</tr>
<tr>
<td><strong>Brf1+Brb2</strong></td>
<td>Middle</td>
<td>3.10</td>
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<td></td>
<td>Lower</td>
<td>5.20</td>
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<td><strong>Cont.</strong></td>
<td>Upper</td>
<td>3.90</td>
<td>ij</td>
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<td>Middle</td>
<td>6.60</td>
<td>d</td>
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<td></td>
<td>Lower</td>
<td>9.87</td>
<td>a</td>
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<tr>
<td><strong>Alt5</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>Brf1</em></td>
<td>Upper</td>
<td>2.20</td>
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<tr>
<td><strong>Brb2</strong></td>
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<td>Lower</td>
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<td><strong>Brf1+Brb2</strong></td>
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<td>Lower</td>
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<td><strong>Cont.</strong></td>
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<td></td>
<td>Middle</td>
<td>5.00</td>
<td>g</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>8.80</td>
<td>b</td>
</tr>
</tbody>
</table>

* 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 × 10⁵ spore/ml of *A. alternata.*

**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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fungal isolate</th>
<th><em>Alt1</em></th>
<th><em>Alt2</em></th>
<th><em>Alt3</em></th>
<th><em>Alt4</em></th>
<th><em>Alt5</em></th>
<th><em>Alt6</em></th>
<th><em>Alt7</em></th>
<th><em>Alt8</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brf1</em></td>
<td>20.00</td>
<td>25.00</td>
<td>16.00</td>
<td>30.00</td>
<td>33.00</td>
<td>21.33</td>
<td>22.00</td>
<td>22.00</td>
<td>30.00</td>
</tr>
<tr>
<td><em>Brb2</em></td>
<td>35.00</td>
<td>28.00</td>
<td>20.00</td>
<td>51.00</td>
<td>40.00</td>
<td>33.00</td>
<td>30.00</td>
<td>38.00</td>
<td>30.00</td>
</tr>
<tr>
<td><em>Cont.</em></td>
<td>78.00</td>
<td>84.00</td>
<td>72.00</td>
<td>64.00</td>
<td>86.67</td>
<td>68.00</td>
<td>82.00</td>
<td>76.00</td>
<td>78.00</td>
</tr>
<tr>
<td><em>LSD</em>&lt;sub&gt;0.05&lt;/sub&gt;</td>
<td>6.21</td>
<td>4.76</td>
<td>4.00</td>
<td>7.74</td>
<td>4.80</td>
<td>6.86</td>
<td>4.76</td>
<td>8.63</td>
<td>4.76</td>
</tr>
</tbody>
</table>

**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.

**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 × 10⁵ spore/ml of *A. alternata.*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fungal isolate</th>
<th><em>Alt1</em></th>
<th><em>Alt2</em></th>
<th><em>Alt3</em></th>
<th><em>Alt4</em></th>
<th><em>Alt5</em></th>
<th><em>Alt6</em></th>
<th><em>Alt7</em></th>
<th><em>Alt8</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brf1</em></td>
<td>43.00</td>
<td>25.33</td>
<td>31.00</td>
<td>53.00</td>
<td>54.67</td>
<td>40.00</td>
<td>38.00</td>
<td>42.00</td>
<td>30.00</td>
</tr>
<tr>
<td><em>Brb2</em></td>
<td>62.67</td>
<td>37.00</td>
<td>34.00</td>
<td>66.00</td>
<td>61.00</td>
<td>48.00</td>
<td>47.00</td>
<td>51.00</td>
<td>30.00</td>
</tr>
<tr>
<td><em>Brf1+Brb2</em></td>
<td>35.00</td>
<td>20.00</td>
<td>24.67</td>
<td>45.00</td>
<td>47.00</td>
<td>33.00</td>
<td>30.00</td>
<td>38.00</td>
<td>30.00</td>
</tr>
<tr>
<td><em>Cont.</em></td>
<td>52.00</td>
<td>55.33</td>
<td>64.00</td>
<td>75.00</td>
<td>78.00</td>
<td>64.00</td>
<td>78.00</td>
<td>81.00</td>
<td>78.00</td>
</tr>
<tr>
<td><em>LSD</em>&lt;sub&gt;0.05&lt;/sub&gt;</td>
<td>8.17</td>
<td>6.50</td>
<td>6.41</td>
<td>5.41</td>
<td>3.69</td>
<td>4.80</td>
<td>4.31</td>
<td>3.39</td>
<td>3.39</td>
</tr>
</tbody>
</table>
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: Brevibacillus1 (Brf1), Brevibacillus (Brb2), mixture of both strains (Brf1+Brb2), and water spray as control treatment. The two Brevibacillus strains 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 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 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].

### 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 Chaetonium 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

![Figure 3: Banding patterns of soluble protein of potato leaves treated by Brevibacillus strains, 0: control, 1:Brf1+Brb2; 2: Brb2; 3:Brf1+Brb2.](image-url)

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

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Treatment</th>
<th>DI Reduction %</th>
<th>DS Reduction %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alt2</strong></td>
<td>Brf1</td>
<td>50.0 mn0</td>
<td>36.0 fg</td>
</tr>
<tr>
<td></td>
<td>Brb2</td>
<td>28.0 ijk</td>
<td>41.7</td>
</tr>
<tr>
<td></td>
<td>Brf1+Brb2</td>
<td>19.3 no</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
<td>48.0 d</td>
<td>0</td>
</tr>
<tr>
<td><strong>Alt4</strong></td>
<td>Brf1</td>
<td>25.0 klm</td>
<td>54.5</td>
</tr>
<tr>
<td></td>
<td>Brb2</td>
<td>31.0 ghij</td>
<td>43.6</td>
</tr>
<tr>
<td></td>
<td>Brf1+Brb2</td>
<td>21.0 lmn</td>
<td>61.8</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
<td>55.0 bc</td>
<td>0</td>
</tr>
<tr>
<td><strong>Alt5</strong></td>
<td>Brf1</td>
<td>18.0 no</td>
<td>66.0</td>
</tr>
<tr>
<td></td>
<td>Brb2</td>
<td>21.0 lmn</td>
<td>60.4</td>
</tr>
<tr>
<td></td>
<td>Brf1+Brb2</td>
<td>15.0 no</td>
<td>71.7</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
<td>53.0 c</td>
<td>0</td>
</tr>
</tbody>
</table>

* Means with the same letter are not significantly different.
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 pathogen. 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 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.

References

1. Food and Agriculture Organization Corporate Statistical Database (2016), Rome, Italy.
key player in sustainable agriculture by improving soil fertility, plant tolerance
as a potential plant growth promoting rhizobacteria for cotton (Gossypium
Alternaria-species on potatoes and tomatoes. Fourteenth Euroblight Workshop,
Germany.
195-218.
genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from
Microbiol 68: 3328-3338.
of Bacillus sp. within vitro antagonistic activity against Fusarium oxysporum
41. Abouvette C, Lemanceau P, Steinberg C (1993) Recent advances in the bio-
42. Coskunntuna A, Özer N (2008) Biological control of onion basal rot disease
using Trichoderma harzianum and induction of antifungal compounds in onion set
mycelial growth of several phytopathogenic fungi by soluble potassium silicate.
of Enysiphe graminis var. tritici. Phytopathol 52: 714.
chitosan on hyphal growth and spore germination of plant pathogenic and
Microbiol 7: 2.
to reduce the variability of biological control. Phytopathol 91: 621-627.
of pathogenesis-related proteins in barley during the resistance reaction to
49. Laemmli UK (1970) Cleavage of structural proteins during the assembly of
in plant against Fusarium wilt through bio-agents as inducers. Afr J Agric Res
7: 5849-5857.
(2nd edn), John Wiley & Sons, Hoboken, New Jersey, USA.
52. Sharma JN, Gupta D, Bhardwaj LN, Kumar R (2005) Occurrence of
Alternaria leaf spot (Alternaria alternata) on apple and its management. Int
53. Babu S (1994) Studies on leaf blight of tomato (Lycopersicon esculentum
Mill.) caused by Alternaria solani (Ell. and Mart.) Jones and Grout. M.Sc. (Ag.)
blight caused by Alternaria palendi-Ayyangar. M.Sc., (Ag.) Thesis, Tamil Nadu
Agricultural University, Coimbatore, India. p. 120.
protein production: From molecular and biochemical fundamentals to
57. Costa RG, Santos NM, De Medeiros AN, De Egypto Queiroga RCR, Madurga
58. Sivanantham T, Rasaiyath V, Satkunathan N, Thavarajan CT (2013) In vitro
screening of antagonistic effect of soil borne bacteria on some selected
59. Viker MHPW, Keizer LCPP, Budding DJ, Van Loon LC, Colon LT, Struk PC
(2003) Leaf position prevails over plant age and leaf age in reflecting resistance
to late blight in potato. Phytopathol 93: 666-674.
60. Mizukami M, Hanagata H, Miyauchi A (2010) Brevibacillus expression system:
Host-vector system for efficient production of secretory proteins. Curr Pharm
61. Goth RW (1997) A detached-leaf method to evaluate late blight resistance in
62. Vteeshouwers VGAA, Van Dooijeweert W, Keizer LCP, Sijpkes L, Govers F, et
al. (1999) A laboratory assay for Phytophthora infestans resistance in various
Benzothiadiazole, a novel class of inducers of systemic acquired resistance,
activates gene expression and disease resistance in wheat. The Plant Cell 8:
629–643.
64. Ishii H, Tomita Y, Horio T, Narusaka Y, Nakazawa Y, et al. (1999) Induced
resistance of acibenzolar-S-methyl (CGA-245704) to cucumber and Japanese
acetylsalicylic acid on B1,3-glucanase activity and disease resistance in
changes in wheat induced by Chaetomium globosum against spot blotch
pathogen. Indian Phytopath 54: 374-379.
68. Romersio RS, Larina Filho R, Vieira Junior JR, Silva HSA, Baracat-Pereira
rhizobacterium as elicitors of systemic resistance in tomato to bacterial and
69. Silva HSA, Romero RS, Macagnan D, Halfeld-Vieira BA, Baracat-Pereira MC,
plants: non-specific protection and increase in enzyme activities. Biological
Control 29:288-295.