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The Antifungal Effects of Four Tomato Rhizosphere *Bacillus* spp. against *Alternaria Alternata*

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Abstract: ***Biocontrol of early blight disease, through the use of natural alternatives to pesticides was studied by using four Bacillus spp. (B. subtilis, B. megaterium, B. pumilus and B. cereus). The antifungal effects of four Bacillus species isolated from tomato rhizosphere were tested against A. alternata (Fr.) Keissl. in vitro and in vivo pot experiment which repeated for two seasons 2007/2008 and 2008/2009. The in vitro growth rates of A. alternata were always lower when it was treated with any of the four Bacillus species in comparison with the untreated control. Statistical analysis revealed that the use of B. subtilis and B. megaterium significantly (P≤ 0.05) suppressed the growth of A. alternata in vitro. In the in vivo pot tests, tomato plants treated with the four Bacillus species displayed suppressed disease incidence and disease severity. This study highlighted the promising effect of the four bacteria species tested in reducing disease incidence and severity in comparison with the control treatment and attempting to include non-toxic bio-agent in an integrated management of early blight disease.***

Keywords: Bacillus subtilis; B. Megaterium; B. Pumilus; B. Cereus; rhizosphere; in vitro; in vivo.

1. Introduction

Early blight epidemics initially progress slowly but accelerate as plants mature, resulting in a typical sigmoidal disease progress curve [11]. Occasionally, the disease curve is bimodal which could be due to the emergence of new healthy leaves after the first cycle of infection [12].Yield losses up to 79% due to early blight damage were reported from Canada, India, USA, and Nigeria [3].

Disease management strategies including rotation with non-host crops and sanitation are not entirely satisfactory since the fungus is primarily air-borne, has long survival ability in plant debris, and has a wide solanaceous host range plants [3]. Fungicide treatments are the most effective way to control the disease to a non-damaging level. Typically, fungicides are applied starting from two weeks after transplanting until two weeks before harvest at two- to three-week intervals. Such heavy use of chemicals is not economically feasible for the generally resources-limited growers. It also imposes health concerns for growers and consumers as well as environmental hazards. In the long run, the intensive use of fungicides could stimulate the emergence of resistant variants of the fungus, as has been reported recently in the USA. Thus, alternative approaches which include biopesticides that can be incorporated into integrated pest management of tomato early blight disease are needed [13].

Biological control agents have been used successfully in some pathogen/host systems to enhance plant growth and control disease. Several mechanisms have been demonstrated. Biological control organisms can act as antagonists or predators of the targeted pest, or by inducing resistance in the host [8].

Plant growth-promoting rhizobacteria are among the various groups of plant-associated microorganisms that can elicit plant defences [7]. They seem to be the best studied organisms, as they have been reported as the responsible for the systemic resistance against plant pathogenic fungi, bacteria, nematodes, and viruses [5]. Induced systemic resistance by using plant growth-promoting rhizobacteria has been demonstrated in many plant species, including rockcress (*Arabidopsis* spp.), bean, carnation, cucumber, radish, tobacco and tomato [16]. [8] studied the effects of some plant growth-promoting rhizobacteria on seedling growth and naturally occurring diseases on tomato in Florida. They used different strains of *Bacillus subtilis*, *B. amyloliquefaciens*, *B. pumilis* and *B. cereus* and found that, all bacterial strains significantly increased plant growth for all parameters measured. But, visual field evaluation ratings for naturally occurring diseases on tomato did not indicate any effect of these bacteria on incidence of *Fusarium* wilt, early blight and tomato yellow leaf curl virus. [1] demonstrated that an isolate of *Bacillus mycoides* obtained from the sugarbeet phylloplane was able to control the leaf spot *Cercospora beticola*. Also 300 prokaryotic phylloplane residents were isolated from healthy tomato plants and tested as candidates to control diseases of the above ground tomato organs. The results proved that one strain (UFV-IEA6) of *Bacillus cereus* was considered the most promising among them [5]. The antifungal activity of *B. subtilis* and *B. licheniformis* was demonstrated *in vitro* by [17] against some sapstaining fungi. They found that, the crude active supernatant fractions of 7 days old *B. subtilis* and *B. licheniformis* cultures inhibited the growth of sapstaining fungi in laboratory experiments. Also, when the antagonistic and inhibitory activities of some *B. subtilis* isolates were tested against the *in vitro* growth of *Rhizoctonia solani*, *Helminthosporium* spp., *Alternaria* spp. and *Fusarium*

oxysporum, the bacterial isolates were found to have strong antagonistic effect on all fungal isolates but they were more effective on *Helminthosporium* spp., *Alternaria* spp. and *F. oxysporum* [9]. In another study, the bacterium *B. megaterium* was found to produce diffusible and volatile compounds that inhibited the growth of *A. alternata* and *F. oxysporum* [15]. In the 1990s, several plant growth-promoting rhizobacteria-based products became commercially available in the United States of America such as AQ10® which is used as a control agent against powdery mildew of fruit, primarily grapes. Also more products are currently under development. Most of these products contain strains of *Bacillus* sp. Earlier attempts to commercialize products containing fluorescent pseudomonad strains of plant growth-promoting rhizobacteria generally failed due to lack of long-term viability of these asporogenous bacteria [4] ,[7].This study was designed to determine the *in vitro* and *in vivo* antifungal effects of some *Bacillus* species for the control of early blight disease in tomato crop.

2. Materials and Methods

The inhibition effect of some bacteria isolated from root rhizosphere of immune tomato plants against early blight disease were assessed *in vitro*. Then a nursery experiment was conducted for two winter cropping seasons (2007/08 and 2008/09) to determine whether the *in vitro* activity of these bacterial isolates will translate into *in vivo* activity. The inhibition activity of these bacterial isolates was evaluated in comparison to the fungicide Ridomil Gold and to control (non-treated tomato plants).
Infected tomato leaves displaying typical symptoms of early blight disease were collected from a sick plot in the University of Khartoum Demonstration Farm (Shambat). Diseased leaves segments were washed thoroughly with tap water and surface sterilized with sodium hypochlorite solution 5.25% and plated onto Potato Dextrose Agar (PDA) medium. Plates were incubated for 7 days at 25°C ±2. Sub-cultures were later prepared to obtain pure cultures.

Soil samples were collected from the rhizosphere of tomato plants that have shown high immune level against early blight disease. Thirty six (36) different bacteria isolates were obtained and purified from soil samples using the soil Dilution Plate Method (Waller et al. 2001). *In vitro* screening test was conducted for these isolates to select bacteria with inhibitory effect against *Alternaria alternata*. Four isolates of *Bacillus* spp. (Ba., Bb., Bc. and Bd.) which showed inhibitory effects against *A. alternata* were selected for the study.

The identification of the selected rhizobacteria was based on the standard biochemical tests [14] used with reference to Bergey`s Manual [2].

For each of the four isolates, a 5-mm-diameter disc from a 7-day old culture of *A. alternata* was inoculated onto PDA medium at the centre of 9-cm-diameter Petri dishes. Then, the bacteria isolates were inoculated separately onto the four edges of each plate 4 cm from where the fungus was inoculated. The plates were incubated at 25ºC ±2, and growth rates were assessed every two days.

The open pollinated tomato variety, Peto 86, which is susceptible to early blight disease was used in this experiment. Seeds were first germinated on damp filter papers in plastic Petri dishes for 3 days.

Bacterial suspensions of the four selected *Bacillus* spp. (Ba., Bb., Bc. and Bd.), that previously showed significant inhibition effect *in vitro* against *A. alternata* in the screening test, were prepared at approximate maximum concentrations of 28 $\times 10^7$ cells/ml. The 3-days-old tomato seedlings were then soaked into the bacterial suspensions for 24 hrs at room temperature. Some other seedlings were soaked in sterile distilled water to serve as untreated control and some other as a fungicide control. After aone-day incubation, the seedlings were transferred to 9-inch diameter plastic pots filled with 7 kg early blight infected soil acquired from a sick plot with tomato diseased debris. Six seedlings were transplanted in each pot and two weeks later thinned to three seedlings/ pot. After six weeks, some of the non-bacterized seedlings were sprayed twice at 15-day interval with Ridomil Gold (1kg/fed.). Each treatment was replicated four times using three pots per replication and arranged in a completely randomized design (CRD).

The disease parameters assessed were: disease incidence which was recorded weekly and disease severity which was recorded once at the end of the growing season. An arbitrary disease severity rating scale of 0- 4 was adopted following [6].

The percentage data were converted to square root and arcsine values [10]. The statistical analysis was accomplished in SAS 9.0 version, and the Duncan`s multiple range test (DMRT) was adopted to compare means. Least significant difference values at $P \leq 0.05$ were used to separate treatment means when ANOVA indicated a significant *F* value.

3. Results

Thirty six bacterial strains were isolated from the rhizosphere of tomato plants showing immune response to early blight disease. Four strains were selected depending on their *in vitro* inhibition activity against *A. alternata* in the screening test. The identification of the selected bacteria was performed depending on the standard biochemical tests (Buchanan 1989; Steubing 1993). The four selected bacteria were identified as different species of the genus *Bacillus* these were; *Bacillus subtilis* Ba., *B. megaterium* Bb., *B. pumilus* Bc., and *B. cereus* Bd. (Table 1).

The highest *A. alternata* growth rate of 0.458mm/hr was recorded for the control treatment 48 hours after commencement of the experiment. The least growth rate of *A. alternata* of 0.240mm/hr was recorded for *B. subtilis* after 216 hours from the commencement of the experiment. The growth rates of *A. alternata* recorded at the end of the experiment showed that there were no obvious differences between the control treatment, *B. pumilus* and *B. cereus*. The statistical analysis revealed that no significant difference was detected between *B. subtilis* and *B. megaterium*. The use of *B. subtilis* and *B. megaterium* significantly ($P \leq 0.05$)

suppressed the growth of *A. alternata in vitro* in comparison with the other treatments (Table 2).

Table 1: Differential characteristics tests of the bacteria species

Biochemical tests	*Bacterial isolates*			
	Ba	*Bb*	*Bc*	*Bd*
Acid fast	—	—	—	—
Shape	Rod	Rod	Rod	Rod
Gram staining	+	+	+	+
Endospore staining	+	+	+	+
Motility test	+	+	+	+
Growth in air	+	+	+	+
Catalase test	+	+	+	+
Glucose (acid)	+	+	+	+
Anaerobic growth	—	—	—	+
V.P test	+	—	+	—
Acid from				
D-glucose	+	+	+	+
L-Arabinose	+	d	+	—
D-xylose	+	d	+	—
D-mannitol	+	d	+	—
Gas from glucose	—	—	—	—
Casein hydrolysis	+	+	+	+
Starch hydrolysis	+	+	—	+
Utilization of citrate	+	+	+	d
Nitrate reduction	+	—	—	—
Indole test	—	—	—	—
Urease test	d	d	—	d
Growth in NaCl				
2%	+	+	+	+
5%	+	+	+	+
7%	+	d	+	d
10%	—	—	—	—
Growth at				
5°C	—	d	—	—
10°C	d	+	+	d
30°C	+	d	+	+
40°C	+	—	+	d
50°C	+	—	—	—
55°C	—	—	—	—
65°C	—	—	—	—
Oxidase test	—	—	—	d

Key: Ba, *Bacillus subtilis*; Bb, *Bacillus megaterium*; Bc, *Bacillus pumilus*; Bd, *Bacillus cereus*; —, 90%or more of the strains are negative; +, 90% or more of the strains are positive; **d**, 11-89% of the strains are positive.

Table 2: Average of growth rates of the fungus *Alternaria alternata* treated with four *Bacillus* spp. *in vitro*

Treatments	Time after culturing (hrs)				
	48hrs	96hrs	144hrs	192hrs	216hrs
Ba.	0.375	0.638	0.480	0.273	0.240
Bb.	0.293	0.668	0.563	0.363	0.318
Bc.	0.375	0.695	0.605	0.435	0.410
Bd.	0.418	0.683	0.668	0.480	0.423
C.	0.458	0.693	0.708	0.488	0.433

Key: Ba, *Bacillus subtilis*; Bb, *Bacillus megaterium*; Bc, *Bacillus pumilus*; Bd, *Bacillus cereus*; C, Control treatment.

In the first season 2007/08, the least disease incidences of early blight of 23.35 and 25.89 were recorded for six-weeks-old plants treated with the fungicide Ridomil Gold and *B. subtilis*, respectively. At the end of the same season, the highest disease incidences of 71.45 and 75.40 were recorded, respectively, for plants treated with *B. cereus* and the control treatment. The statistical analysis revealed no significant differences among the treatments (Table 3). In the second season 2008/09 the least disease incidences of 27.72 and 28.33 were recorded for plants treated with *B. subtilis* and Ridomil Gold respectively when the age of the plants was six weeks.

Table 3: Disease incidence of tomato plants treated with different *Bacillus* spp. and the fungicide Ridomil Gold® in season 2007/08

Treatments	Plants age (weeks)			
	6	7	8	9
Ba.	25.89^{a}	35.58^{b}	49.03^{a}	59.85^{a}
Bb.	31.31^{a}	41.73^{ab}	61.92^{a}	66.58^{a}
Bc.	29.49^{a}	43.44^{ab}	62.11^{a}	63.73^{a}
Bd.	38.54^{a}	48.23^{ab}	69.29^{a}	71.45^{a}
F.	23.35^{a}	37.55^{b}	49.24^{a}	57.84^{a}
C.	39.98^{a}	53.44^{a}	62.63^{a}	75.40^{a}

Key: Ba, *Bacillus subtilis*; Bb, *Bacillus megaterium*; Bc, *Bacillus pumilus*; Bd, *Bacillus cereus*; C, Control treatment; F., Fungicide Ridomil Gold.*Percentage data were transformed to Arc sine.**Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan` s Multiple Range Test (DMRT).

In the same season, when the age of the plants was 9 weeks, the least disease incidences of 45.80 and 53.34 were recorded for the same treatments, respectively. The highest disease incidences of 43.57, 59.63, 68.99 and 77.42 were recorded for the control treatment throughout the season which were significantly ($P \leq 0.05$) higher than the other treatments (Table 4).

Table 4: Disease incidence of tomato plants treated with different *Bacillus* spp. and the fungicide Ridomil Gold® in season 2008/09

Treatments	Plants age (weeks)			
	6	7	8	9
Ba.	27.72^{b}	42.39^{b}	45.43^{b}	45.80^{b}
Bb.	34.57^{ab}	43.58^{b}	48.92^{b}	63.92^{ab}
Bc.	30.52^{b}	40.97^{bc}	45.60^{b}	53.83^{ab}
Bd.	29.74^{b}	40.11^{bc}	45.54^{b}	60.54^{ab}
F.	28.33^{b}	32.64^{c}	42.42^{b}	53.34^{ab}
C.	43.57^{a}	59.63^{a}	68.99^{a}	77.42^{a}

Key: Ba, *Bacillus subtilis*; Bb, *Bacillus megaterium*; Bc, *Bacillus pumilus*; Bd, *Bacillus cereus*; F., Fungicide Ridomil Gold; C.,Control treatment.*Percentage data were transformed to Arc sine.**Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan` s Multiple Range Test (DMRT).

The least disease severity of 24.85 and 25.39 were recorded at the end of the first and second seasons, respectively, from tomato plants sprayed with Ridomil Gold. The highest disease severity of 31.42 and 39.63 were recorded in the first and second seasons, respectively, for plants treated with *B. cereus* and the control treatment. Statistical analysis revealed that the use of Ridomil Gold, *B. subtilis* and *B. megaterium* significantly ($P \leq 0.05$) reduced disease severity in the first season. But no significant differences among the treatments were observed in the second season (Table 5).

Table 5: Disease severity of tomato plants treated with different Bacillus spp. And the fungicide Ridomil Gold® in seasons 2007/08 and 2008/09

Treatments	2007/08	2008/09
Ba.	25.91[b]	29.71[a]
Bb.	27.07[ab]	30.85[a]
Bc.	27.86[ab]	30.80[a]
Bd.	31.42[a]	34.74[a]
F.	24.85[b]	25.39[a]
C.	31.21[a]	39.63[a]

Key: Ba, *Bacillus subtilis*; Bb, *Bacillus megaterium*; Bc, *Bacillus pumilus*; Bd, *Bacillus cereus*; F., Fungicide Ridomil Gold; C.,Control treatment.*Percentage data were transformed to Arc sine.**Means followed by the same letter are not significantly different at $P \leq 0.05$, according to Duncan` s Multiple Range Test (DMRT).

4. Discussion

The selection of the four bacteria species used in this experiment was based on their *in vitro* ability to inhibit the growth of the fungus *Alternaria alternata*. These were *B. subtilis*, *B. megaterium*, *B. pumilus* and *B. cereus*. This identification was based on the recommended standard biochemical tests for bacterial identification [2], [14].

Inhibition effects of these four *Bacillus* spp. on growth of *A. alternata* were assessed *in vitro* under laboratory conditions. The inhibition effects of the four species varied; some showed satisfactory inhibition effects, while others showed little or no inhibition effects and were not different from the control treatment. Plates of *A. alternata* which were inoculated with *B. subtilis* and *B. megaterium* showed significantly less mycelial growth during the incubation period and this finding agreed with the reports by [15], [9] and [17], who indicated the ability of these bacteria to inhibit *in vitro* mycelial growth of many plant pathogenic fungi. No significant differences in the inhibition of *A. alternata* were observed between the species of *B. pumilus*, *B. cereus* and the control treatment.

The *in vitro* inhibitory activity of these bacteria species translated well into the *in vivo* test; *B. subtilis* and *B. megaterium* showed the highest inhibition effect *in vitro* against mycelial growth of the causative agent of early blight disease in tomato. Their effects in *in vivo* test on early blight disease incidence were also the best. On the other hand, *B. pumilus* showed low and unsatisfactory *in vivo* effect when compared with Ridomil Gold, which is agreed with the *in vitro* results, and with findings of [8]. The fourth species *B. cereus* showed low suppressive effects against disease incidence during both seasons. These results agreed with the laboratory findings and the findings published by [8]. But are incompatible with the findings of [5] who studied the control effect of 300 prokaryotic phylloplane residents against diseases of the above ground tomato organs, and the results proved that *Bacillus cereus* was considered to be the most promising among them.

The suppression effect of these bacteria on disease severity did not differ from their effect on disease incidence. The lowest percentages of disease severity were recorded at the end of both seasons from tomato plants sprayed with Ridomil Gold. The highest disease severity was recorded for the control treatment in both seasons. The control effect of *Bacillus* spp. on disease severity was moderate and placed in the middle between the fungicide and control treatments.

The intensive use of fungicides, the emergence of resistant pathogen variant and the negative impact on human health and environment, when take these situations into account; This study highlighted the promising effect of the four bacteria species tested in reducing disease incidence and severity in comparison with the control treatment and attempting to include non-toxic bio-agent in an integrated management of early blight disease.

References

[1] Bargabus, R.L.; Zidack, N.K.; Sherwood, J.E. and Jacobsen, B.J. (2004). Screening for the identification of potential biological control agents that induce systemic acquired resistance in sugar beet. *Journal of Biological Control* **30**, 342-350.
[2] Buchanan R E (1989) *Bergey's Manual of Systemic Bacteriology*. Volumes I-IV. Wilkins and Wilkins, Baltimore. 2648pp.
[3] Chaerani R, Voorrips R E (2006) Tomato early blight (*Alternaria solani*): the pathogen, genetics, and breeding for resistance. *J. Gen. Plant Pathol*. 72: 335-347.
[4] Franklin R H, Julius J M (1999) (eds.). *Biopesticides: Use and delivery*. Human Press Inc. 619pp.
[5] Halfeld-Vieira A B, Vieira J R, Romeiro R S, Silva H S, Baracat P M (2006) Induction of systemic resistance in tomato by the autochthonous phylloplane resident *Bacillus cereus*. *Pesqu. Agropecuária Brasileira* 41(8): 1247-1252.
[6] Horsfall J G, Barratt R W (1945) An improved grading system for measuring plant diseases. *Phytopathology* 35: 655.
[7] Kloepper J W, Ryu C M, Zhang S (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94: 1259-1266.
[8] Kokalis-Burelle N, Vavrina C S, Rosskopf E N, Shelby R A (2002) Field evaluation of plant growth-promoting rhizobacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. *Plant Soil* 238: 257-266.
[9] Matar S I, El-Kazzaz, S A, Wagih E E, El-Diwany A I, Moustafa H E, Abo-Zaid G A, Abd-Elsalam H E. Hafez E E (2009) Antagonistic and inhibitory effect of *Bacillus subtilis* against certain plant pathogenic fungi, I. *Biotechnology* 8(1): 53-61.
[10] McDonald J H (2009) *Handbook of Biological Statistics*. 2nd edition. Sparky House Publishing, Baltimore, Maryland. 293pp.
[11] Nash A F, Gardner R G (1988) Tomato early blight resistance in a breeding line derived from *Lycopersicon hirsutum* PI 126445. *Plant Dis*. 72: 206-209.
[12] Pandey K K, Pandey P K, Kallo G, Banerjee M K (2003) Resistance to early blight of tomato with respect to various parameters of disease epidemics. *J. Gen. Plant Patho*. 69: 364-371.

[13] Pasche J S, Wharam C M, Gudmestad N C (2004) Shift in sensitivity of *Alternaria solani* to Q_oI fungicides. *Plant Dis*. 88: 181-187.
[14] Steubing P M (1993) Isolation of an unknown bacterium from soil. Pages 81-114, In: *Tested Studies for Laboratory Teaching*. Ed. Goldman, C.A. Volume 14 Proceedings of the 14th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 240pp.
[15] Trivedi P, Pandey A (2008) Plant growth promotion abilities and formulation of *Bacillus megaterium* strain B 388 (MTCC 6521) isolated from a temperate Himalayan location. *Indian J. Microbiol*. 48(3): 342-347.
[16] Van Loon L C, Bakker P A, Pieterse C J (1998) Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol*. 36: 453-483.
[17] Velmurugan N, Choi M S, Han S S, Lee Y S (2009) Evaluation of antagonistic activity of *Bacillus subtilis* and *Bacillus licheniformis* against wood staining fungi: *in vitro* and *in vivo* experiments. *J. Microbiol*. 47(4): 385-392.
[18] Waller J, Lenné J, Waller S (2001) (eds.). *Plant Pathologist` s Pocketbook*. 3rd edition. CABI Publishing, Wallington, UK. 528pp.

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