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Evaluation of rhizosphere bacterial antagonists for their potential to bioprotect potato (*Solanum tuberosum*) against bacterial wilt (*Ralstonia solanacearum*)

Naser Aliye [a,*], Chemeda Fininsa [b], Yaynu Hiskias [c]

[a] National Plant Protection Research Centre, P.O. Box 37, Ambo, Ethiopia
[b] Department of Plant Sciences, Haramaya University, P.O. Box 138, Dire Dawa, Ethiopia
[c] Institute of Biodiversity Conservation, P.O. Box 30726, Addis Ababa, Ethiopia

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ABSTRACT

Bacterial wilt (*Ralstonia solanacearum*) is one of the production constraints of potato (*Solanum tuberosum*). The intent of the study was to evaluate potential of bacterial antagonists to suppress bacterial wilt disease development and evaluate the role of the strains as plant growth-promoting rhizobacteria (PGPR) in potato. One hundred-twenty rhizosphere bacterial isolates were screened against virulent strain of *Ralstonia solanacearum* PPRC-Rs. After *in vitro* screening, six antagonistic strains (PFMRI, BS-DFS, PF9, PF20, BC, and BS-wly) with inhibition diameter >11 mm were selected and studied further in the greenhouse, *in vivo*. During *in vivo* study, the strains were evaluated for their effect in suppressing disease development in terms of area under disease progress curve (AUDPC) and increasing biomass (plant height and dry weight) of potato. Accordingly, PFMRI, BS-DFS, and PF9, significantly reduced AUDPC by 78.6, 66, and 64.3%, and wilt incidence by 82.7, 66.2, and 65.7%, respectively, compared to the control. During the sole application, the strains significantly ($P < 0.0001$) increased plant height by 35.6, 45.9, and 45%, and dry matter by 111, 130.4, and 129%, respectively compared to non-bacterized control. In the presence of the pathogen strain PFMRI, BS-DFS, and PF9 increased plant height by 66, 50, and 48.2%, and dry matter by 153.8, 96.8, and 92.5%, respectively compared to the pathogen treated control. Hence, the study shows that PFMRI, BS-DFS, and PF9 strains have potential use in potato bioprotection, as PGPR or in an integrated bacterial wilt management; whose effectiveness under a variety of field conditions should be investigated.

1. Introduction

Potato (*Solanum tuberosum* L.) is one of the most widely used crops worldwide. International Potato Centre (CIP) estimated that potato production in developing countries is expanding at the rate of 2.8% annually (CIP, 1995). The crop is an important income source for farmers in the mid to highland parts of Ethiopia. The current area under potato production is about 51698 hectares and the average national yield is 9.9 tons ha^{-1} (MoARD, 2005). The national average yield of Ethiopia, however, is very low as compared to the potato yields of other countries of the world (Nganga, 1982). The potential attainable average yields of the crop on research field is 40 tons ha^{-1} and 20 tons ha^{-1} on farmers' field (MoARD, 2005). The gap between the production potential and the current average national production could be attributed to different factors, among which diseases are the most important ones. Potato is susceptible to a number of diseases, including late blight caused by *Phytophthora infestans* Mont Debary, several viruses and bacterial wilt caused by *Ralstonia solanacearum* (Smith). Bacterial wilt is the most destructive in the mid altitude areas around Shashamane, in the Rift Valley, Bako, Jimma, and many irrigated fields through out Ethiopia (Yaynu, 1989; Lemessa and Zeller, 2007).

Plant growth-promoting bacteria (PGPB) (Bashan and Holguin, 1998) are associated with many plant species and are commonly present in many environments. The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and closely adhering soil interface, the rhizosphere (Kloepper et al., 1999). The extent of endophytic colonization of host plant organs and tissues reflects the ability of bacteria to selectively adapt to these specific ecological niches (Hallman et al., 1997; Gray and Smith, 2005). Consequently, intimate associations between bacteria and host plants can be formed without harming the plant (Hallman et al., 1997; Kloepper et al., 1999).

Certain bacteria trigger a phenomenon known as Induced Systemic Resistance (ISR) phenotypically similar to systemic acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive

* Corresponding author. Fax: +251 11 2362325.
E-mail address: anaser22@yahoo.com (N. Aliye).

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reaction through which it becomes limited in a local necrotic lesion of brown, desiccated tissue (Van Loon et al., 1998). As SAR, ISR is effective against different types of pathogens but differs from SAR in that the inducing PGPB does not cause visible symptoms on the host plant (Van Loon et al., 1998). Biopriming plants with some PGPB can also provide systemic resistance against a broad spectrum of plant pathogens (Compant et al., 2005). Diseases of fungal, bacterial, and viral origin and in some instances even damage caused by insects and nematodes can be reduced after application of PGPR (Sturz et al., 2000).

The common control measures used against bacterial wilt include the use of resistant varieties, healthy seed, crop rotation, agronomic practices, biological control and integrated management (Elphinstone and Aley, 1993). However, there are no universal control measures which are effective across the wide host range of the pathogen (Cook et al., 1989). A control measure that is effective in one particular environment may not necessarily be recommended for another environment. Thus, it is imperative to find management options that could be effective in a specific environment of interest. Although there is a potential for managing the disease using biological agents, no comprehensive work has been attempted under Ethiopian conditions. Therefore, the objectives of the study were to evaluate bacterial antagonists against bacterial wilt of potato, and the effect of the bacterial antagonists on potato plant biomass and survival.

2. Materials and methods

2.1. Description of the study area

Laboratory and greenhouse studies were conducted at the National Plant Protection Research Centre (PPRC), Ambo, Ethiopia, during October 2005–November, 2006. Ambo is located at 8°57′N, 37°52′E, and 2200 masl. The greenhouse was adjusted for its temperature and relative humidity using Air Conditioner and Humidity Control. Data on temperature and relative humidity were collected five times daily (6:00 AM, 9:00 AM, 12:00 AM, 3:00 PM, and 6:00 PM) during the experimental period. The mean maximum and minimum temperatures and relative humidity recorded during the period of each study in the greenhouse were 30.5/22 °C and 72.4/45.2% for the first, 32.5/25 °C and 70.4/40.2% for the second study, respectively.

2.2. Additional Isolates of R. solanacearum, Bacills spp. and Pseudomonas fluorescens used in the study

Ralstonia solanacearum PPRC-Rs, the virulent strain, which belongs to race-3 biovar-2 of *R. solanacearum* (Naser et al., 2007) was used for both *in vitro* and *in vivo* studies. Strains of *Bacillus* spp. (TLI-1 and TLI-2) and *Pseudomonas fluorescens* (PR-4-I-x and PR3-I-x), which were reported to have potential to suppress growth of *R. solanacearum* (Naser and Tesfaye, 2005) were included in the study. The rest of the new rhizobacteria were collected and isolated as described below.

2.3. Soil sample collection and isolation of rhizosphere bacteria

2.3.1. Soil sample collection

Based on the information provided by the farmers, soil samples were collected from three types of fields where *R. solanacearum* was known to exist but its occurrence was low, and where the pathogen was introduced but not established. In certain cases where the history of the field and disease occurrence was not available, biologically active soil (like forest soil, decomposed organic matter or compost) expected to contain a diverse microorganism population were sampled. Soil samples were collected to a 5 cm depth (since that is the root zone for most crops) in the upper profile. Soil samples were collected after plants were dug out carefully with roots and gently shaken to discard excess soil. Any aggregates were removed and discarded, leaving only soil, which was adhering closely to the root system.

One hundred-five soil samples were collected and 500 bacterial isolates were obtained from 10 crops [potato, tomato, pepper, eggplant, maize, enset (*Ensete ventricosum*), banana, sugarcane, 'Chat' (*Chata edulis* Forsk.), and papaya] from various locations of Ethiopia.

The altitude of sampled localities ranged from 1655 masl (Ziway, East Shewa) to 2775 masl (Chencha, Arbaminch). The collected samples were placed in plastic bags and kept in an icebox during field survey and sample collection, and finally kept at 4 °C in the laboratory until processed.

2.3.2. Isolation of rhizosphere bacteria

The bacteria from rhizosphere soil were isolated using nutrient agar (NA), King's B-medium (KB-medium) and potato dextrose agar (PDA) (Dhingra et al., 1995). Ten-gram rhizosphere soil sample in sterile flask with 90 ml water was stirred using magnetic stirrer for 5 min. While the suspension was in motion, 10 ml was withdrawn and added to 90 ml sterile water in a screw-cap flask and was shaken for 1 min, and 10 ml of the suspension was transferred to 90 ml sterile water blank. The process was repeated until dilution of 1:1000 ppm. The dilution was made in amended sterile water. Initially soil samples were shaken in 0.2% NaCl plus 0.05% Na_2CO_3 to increase bacterial count (Dhingra et al., 1995).

One mililiter of the dilution was spread on suitable agar surface by an inclined rotary motion of the plate. For estimation of population on culture media, the plates were prepared 2–3 days before use. In addition, to isolate the spore-forming bacteria selectively, soil suspension was prepared to desirable dilution level. The suspensions in test tubes were put in water bath and heated for 25 min at 85 °C to kill the vegetative cells. After cooling to ~50 °C the suspension was inoculated on NA.

2.4. Biological control of potato bacterial wilt

2.4.1. Preliminary in vitro screening of rhizosphere bacteria for antagonistic activity

The virulent pathogenic isolate of *R. solanacearum* PPRC-Rs (Naser et al., 2007) and pure cultures of rhizosphere bacteria incubated for 48 h were used. Preliminary *in vitro* screening for antagonistic activity against *R. solanacearum* was made using cross inoculation method in which the rhizosphere bacteria was inoculated transversally along the diameter of the plate, and the pathogen at right angle to the rhizosphere bacteria in dual culture medium (Hartman et al., 1993; Dhingra et al., 1995). Accordingly, those bacteria that showed positive *in vitro* inhibitory activity during preliminary screening were selected as 'antagonistic bacteria'.

2.4.2. Laboratory (in vitro study)

To further study the antagonism, systematically, nutrient glucose agar (for all antagonists except for *P. fluorescens*) was seeded with strain PPRC-Rs of *R. solanacearum* by evenly spreading 0.1 ml of the suspension (~10^8 cfu/ml). Paper disc (10 mm) was immersed in each test antagonist and was spotted at the centre of the pathogen-inoculated-plate. Paper disc immersed in distilled sterile water and spotted at the centre of the plates with the pathogen was used as control. The same method was followed for *P. fluorescens* isolates on KB-medium. Completely randomized design (CRD) with four replications was used. Average diameter of the inhibition zones after 72 h of incubation at 30 °C was used as a measure of antagonism (Dhingra et al., 1995). The study was re-

peated twice. The potential bacterial antagonists that showed strong (11–20 mm inhibition diameter) and very strong (over 20 mm inhibition diameter) degree of inhibition (Arsenijevic et al., 1998) were labeled and maintained separately. The most effective *in vitro* inhibitors (>11 mm inhibition diameter) were further evaluated in the greenhouse, *in vivo*.

2.4.3. Greenhouse (in vivo) study

The greenhouse pots were filled with soil mixture prepared from field soil, compost, and sand at the ratio of 2:1:1, respectively. The soil was autoclaved at 121 °C for 2 h. Three kilogram sterile soil was filled into each pot (of 20 cm diameter) into which two eyepieces of potato variety, *Tolcha* (highly susceptible to bacterial wilt) (Naser, 2008) was planted. After 1 month, plants were inoculated with strain PPRC-Rs of the pathogen by the stem-injection method (Dhingra et al., 1995) to refresh the virulence of the pathogen and to infest the soil. Plants were removed after complete wilting, and soil was drenched with the pathogenic bacterial suspension ($\sim 10^8$ cfu/ml) at the rate of 250 ml/pot to ensure complete infestation.

Aqueous suspensions ($\sim 10^8$ cfu/ml) of all the antagonists were prepared from 48-h-old pure cultures grown on NA. Potato eyepieces were removed from sprouted tubers and dipped in suspensions of test organisms, then air-dried and planted in pots infested with *R. solanacearum* (Shekhawat et al., 1993). Four pots each having three treated potato eyepieces per test organism were kept in RCBD and eyepieces dipped in distilled sterile water were planted as a control. The experiment was repeated twice.

Effects of the antagonists on plant height, fresh, and dry weight and wilt reduction were assessed. After potato plant reached its full maturity, plants were measured for their height and then cut just above ground into pieces. The fresh weight data was taken by weighing the above ground part, and the cuts were then oven-dried at 60 °C for 72 h and dry weight was recorded. Plant height and dry weight were used for comparison of bacterial antagonists for their growth promotion effect.

In order to clearly see the relative effect of the antagonists on plant biomass as compared to the non-treated control or treated only with the pathogen, separate evaluation was made for bacterial antagonists when applied alone and with the pathogen. The relative growth promotion efficacy by the bacterial antagonist was calculated as follows:

$$\mathrm{GPE} = [(G_T - G_C)/G_C] \times 100$$

where, GPE is growth promotion efficacy, G_T is growth promotion by the treatment group and G_C is growth by the control.

2.5. Identification of selected bacterial isolates

Identification was made at the University of Bonn, Germany, for the selected bacterial antagonists using Gas Chromatography-Fatty Acid Methyl Esters (GC-FAME) technique according to Sasser (2001). The isolates were cultured on Trypticase Soy Broth Agar (TSBA) and incubated at 28 °C for 24 h before saponification, methylation, and purification of FAMEs. The purified esters were then separated using a Hewlet–Packard Model 6890 gas chromatograph equipped with a 25-m fused silica capillary column using nitrogen as the carrier gas. The separated esters were identified with known standards using a microbial identification system software package, MIDI (Microbial ID, Inc., New Jersey, USA) based on actual calibration retention times run prior to sample analysis.

2.6. Data collection

In the laboratory (*in vitro*) study, diameter of inhibition zone (mm) was measured by taking the average diameter of inhibition zones recorded in the four petridishes for each antagonist. In the greenhouse (*in vivo*) study, wilt incidence (%), percent survival (%), plant height (cm), fresh, and dry weight (g) were recorded. Wilt incidence was recorded by counting the proportion of wilted plants to total plants per pot. The AUDPC was calculated according to the mid point rule (Garrett and Mundt, 2000) as:

$$\mathrm{AUDPC} = \sum_{i=1}^{n-1} 0.5(x_i + x_{i+1})][t_{i+1} - t_i],$$

where x_i is the percentage of disease incidence at ith assessment, t_i is the time of the ith assessment in days from the first assessment date and n is the total number of days disease was assessed. Because incidence (x) was expressed in percent and time (t) in days, AUDPC was expressed in %-days (Campbell and Madden, 1990).

Reduction of wilt incidence and AUDPC was calculated using 'PR = $[(P_C - P_T)/P_C] \times 100$'; where, PR is percent reduction, P_C is percentage value of the control, and P_T is percentage value of the treatment group.

Survival was recorded by counting the proportion of surviving plants to total plants per pot at full maturity and plant height was recorded by measuring the above ground part of the plant after its full maturity. Fresh weight was measured by weighing the above ground cuts of the fully matured plant. Dry matter of plants was measured by weighing the oven-dried (at 60 °C for 72 h) above ground cuts of the fully matured plants.

2.7. Statistical analysis

SAS statistical software version 8 (SAS Institute, 1999) was used for analysis. The data of the two experiments were pooled after variance homogeneity was confirmed using the HOVTEST = LEVENE option of ANOVA procedure. Whenever ANOVA detected significant difference between treatment means, Duncan Multiple Range Test (DMRT) was used for mean comparison. For correlation analysis PROC CORR option of ANOVA procedure was used. A significant level of $\alpha = 0.05$ was used in all analyses.

3. Results

3.1. In vitro study

During preliminary screening of one hundred-twenty rhizosphere bacterial isolates, only eight (BS-JM, BS-2ab, PFMRI, BS-wly, BS-DFS, BC, PF9, and PF20) were found to inhibit growth of the pathogen *in vitro*, all strains were isolates of Plant Protection Research Centre (PPRC). These eight bacterial isolates were selected as 'bacterial antagonists'. Out of the eight antagonistic bacterial isolates, *Bacillus subtilis* PFMRI, *Paenibacillus macerans* BS-DFS, *Paenibacillus macerans* PF9, *P. fluorescens* PF20 were found to inhibit growth of the pathogen significantly ($P < 0.0001$) with mean inhibition diameter of 22.18, 18.60, 17.58, and 14.15 mm (Table 1), respectively.

The *Bacillus* sp. (strain TLI-1 and TLI-2), *P. fluorescens* (strain PR-3-I-x and PR-4-I-x) BS-JM, and BS-2ab were weak for inhibiting growth of the pathogen *in vitro*, as their mean inhibition diameters were <5 mm. The six isolates (PFMRI, BS-wly, BS-DFS, BC, PF9, and PF20) with >11 mm of inhibition diameter were further studied in the greenhouse (*in vivo*).

3.2. Greenhouse (in vivo) study

During evaluation of the antagonists *in vivo* for bioprotection of potato plant in the greenhouse, no significant difference was observed among treatments up until 50 days after treatment (Fig. 1). At the 50 days after treatment *B. subtilis* PFMRI, *P. fluorescens*

Table 1
Inhibition of *R. solanacearm* colony growth on NA and KB medium, mean diameter of inhibition zone and degree of antagonism of the bacterial antagonists

Strain	Bacterial species	Mean diameter of inhibition zone (mm)[x]	Degree of antagonism[y]
TLI-1	*Bacillus* sp.	3.0250i	+
TLI-2	*Bacillus* sp.	2.4250j	+
PR-3-I-x	*P. fluorescens*	3.5500gh	+
PR-4-I-x	*P. fluorescens*	3.2000hi	+
BS-JM	*K. pneumoniae*	3.6000gh	+
BS-2ab	*K. pneumoniae*	4.0125g	+
PFMRI	*B. subtilis*	22.1750a	+ + + +
BS-wly	*S. marcescens*	12.7250e	+ + +
BS-DFS	*P. macerans*	18.6000b	+ + +
BC	*B. pumilus*	11.4375f	+ + +
PF9	*P. macerans*	17.5750c	+ + +
PF20	*P. fluorescens*	14.1500d	+ + +
PPRC-Rs + DSW[z]	*R. solanacearum*	0.0000	–
CV (%) = 3.56			

[x] Values in the same column followed by the same letter(s) are not significantly different at ($\alpha = 0.05$) according to Duncan Multiple Range Test.
[y] Weak '+' (width of inhibited growth area 1–5 mm), medium '+ +' (6–10 mm), strong '+ + +' (11–20 mm), very strong '+ + + +' (over 20 mm) (Arsenijevic et al., 1998).
[z] The control (distilled sterile water). The nutrient agar (NA) was used for all isolates except for *P. fluorescens* isolates where King's B-medium (KB) was used.

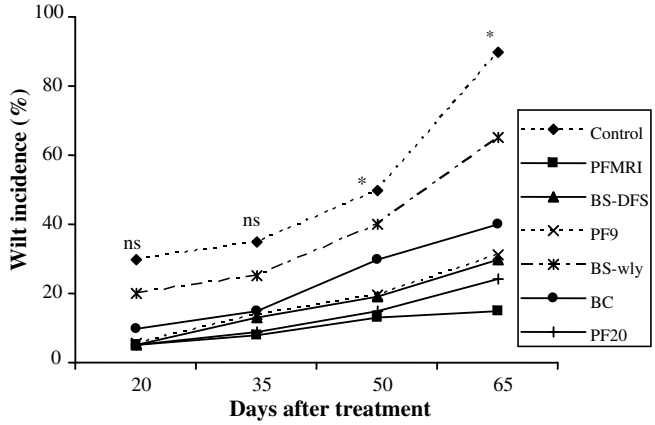

Fig. 1. Bacterial wilt disease progress (symptom development) expressed as wilt incidence on potato cultivar 'Wochecha' treated by different antagonistic bacterial strains (*Bacillus subtilis* PFMRI, *Paenibacillus macerans* BS-DFS and PF9, *Serratia marcescens* BS-wly, *Bacillus pumilus* BC, and *Pseudomonas fluorescens* PF20) after being treated with the bacterial wilt pathogen *Ralstonia solanacearum* PPRC-Rs (250 ml/pot of approximately 10^8 cfu/ml pathogen suspension added to each pot). Antagonists were applied by dipping seeds in aqueous suspensions (10^8 cfu/ml). Control seeds were treated with water only. The treated plants were grown in greenhouse for 70 days at maximum/minimum temperature and relative humidity of 31.5/23.5 °C and 71.4/42.7%, respectively. ns and * indicate absence and presence of significant differences (Duncan Multiple Range Test, $\alpha = 0.05$) among treatments at a particular day after treatment, respectively.

PF20, *P. macerans* BS-DFS, and PF9 significantly ($P < 0.0001$) reduced wilt incidence by 74, 70, 62, and 61%, respectively (Fig. 1) and at 65 days after treatment the same trend was repeated, where strain PFMRI has caused the maximum wilt reduction (Table 2).

Antagonistic strains significantly differed for their ability to suppress the disease development as expressed by AUDPC. Plants treated with *B. subtilis* PFMRI, *P. fluorescens* PF20, *P. macerans* BS-DFS, and PF9 maintained significantly lower AUDPC ($P < 0.0001$), relative to the control (Table 2).

In addition, applications of the bacterial antagonists following inoculation with the bacterial wilt pathogen have increased the survival of potato against bacterial wilt. Thus, application of antagonistic strains like *B. subtilis* PFMRI, *P. fluorescens* PF20 and *P. macerans* BS-DFS, and PF9 increased plant survival from 8.33% without the antagonist (the control) to more than 83%, respectively, after application (Table 2).

The *in vivo* effect of antagonistic bacterial isolates was studied under green house conditions. There were significant differences among treatments for both plant height and dry weight of potato plant when antagonists were applied alone and following application of the pathogen. Accordingly, application of *P. macerans* BS-DFS, and PF9, *B. subtilis* PFMRI, *P. fluorescens* PF20 alone, significantly ($P < 0.0001$) increased plant height and dry matter by more than 33 and 110%, respectively. Likewise, application of the mentioned strains following the application of the pathogen significantly ($P < 0.0001$) increased plant height and dry matter by more than 48 and 92%, respectively as compared to the control (Table 3).

However, *Serratia marcescens* BS-wly was the least successful antagonist in bioprotecting the crop against *R. solanacearum* *in vivo*, as evidenced by the lowest value of wilt reduction, percent survival, and biomass increase, despite its strong inhibition

Table 2
Effect of applying bacterial antagonists on relative survival, reduction of wilt and area under disease progress curve (AUDPC) of potato crop planted in bacterial wilt sick pot under greenhouse conditions

Treatment	Bacterial species	Survival (%)[x]	Reduction of wilt (%)[y]	AUDPC (%-days)	Reduction of AUDPC (%)
PFMRI	*B. subtilis*	100.00a	82.7	465g	78.6
BS-DFS	*P. macerans*	83.34ab	66.2	742.5e	66.0
BS-wly	*S. marcescens*	33.33c	27.7	1612.5b	26.0
PF9	*P. macerans*	83.34ab	65.7	775.5d	64.3
BC	*B. pumilus*	66.67b	49.6	1050c	51.7
PF20	*P. fluorescens*	91.67a	73.0	577.5f	73.4
Control (PPRC-Rs)	*R. solanacearum*	8.33d	–	2175a	–
No bacterization	–	100.00a	–	–	–

[x] Means within columns followed by the same letter(s) are not significantly different at ($\alpha = 0.05$) using Duncan Multiple Range Test.
[y] Reduction of wilt incidence and AUDPC was calculated based on the mean wilt incidence of 90.25% and AUDPC of 2175%-days of the pathogen-inoculated-control pots, using 'PR = $[(P_C - P_T)/P_C] \times 100$'; where, PR is percent reduction, P_C is percentage value of the control, and P_T is percentage value of the treatment group.

Table 3
Growth promotion effect (GPE) of applying bacterial antagonists alone, and in the presence of *R. solanacerum* on biomass of potato plant grown under greenhouse conditions

Treatment	Bacterial species	Plant height (cm)[x]	GPE (%)	Fresh weight (g)[x]	GPE (%)[z]	Dry matter (g)[x]	GPE (%)
Bacterial antagonist alone							
PFMRI	*B. subtilis*	54.250b	35.6	62.600b	114.4	8.500b	111.0
BS-DFS	*P. macerans*	58.375a	45.9	67.775a	132.0	9.275a	130.4
BC	*B. pumilus*	49.875c	24.7	60.050c	105.7	8.038c	99.7
BS-wly	*S. marcescens*	40.000g	—	30.475h	4.4	4.175fg	3.7
PF9	*P. macerans*	58.000a	45.0	68.450a	134.4	9.218a	129.0
PF20	*P. fluorescens*	53.500b	33.8	62.625b	114.5	8.475bc	110.6
Bacterial antagonist + R. solanacearum							
PFMRI + PPRC-Rs		46.500d	66.0	43.175d	—	5.900d	153.8
BS-DFS + PPRC-Rs		42.000e	50.0	33.750g	—	4.575e–g	96.8
BS-wly + PPRC-Rs		34.375h	22.8	30.613h	—	4.150fg	78.5
PF9 + PPRC-Rs		41.500fg	48.2	33.175g	—	4.475e–g	92.5
BC + PPRC-Rs		43.250e	54.5	34.450f	—	4.675ef	101.0
PF20 + PPRC-Rs		43.500e	55.4	35.525e	—	4.825e	107.5
The controls							
PPRC-Rs	*R. solanacearum*	28.000i	—	—[z]	—	2.325h	–
No bacterization (DSW)[y]	—	40.000g	—	29.200i	—	4.025g	–
CV (%)		2.7		5.7		6.7	

GPE was calculated based on 'GPE = $[(G_T - G_C)/G_C] \times 100$'; where, GPE is growth promotion efficacy, G_T is growth promotion by the treatment group, and G_C is growth by the control.

[x] Values in the same column followed by the same letter(s) are not significantly different at ($\alpha = 0.05$) according to Duncan Multiple Range Test.

[y] Distilled sterile water (DSW) only.

[z] As the whole plant in all four pots except one plant died the fresh weight data could not be taken at the end of the experiment and hence, GPE could not be calculated for antagonistic strains applied in the presence of the pathogen as it is calculated based on the value of the pathogen-inoculated-control.

of the pathogen *in vitro*. This indicates the general lack of correlation between *in vitro* antagonism and *in vivo* bioprotection. Generally, there was insignificant ($P = 0.1296$) correlation of *in vitro* inhibition in the laboratory and *in vivo* biocontrol in the greenhouse as examined by analyzing the correlation of values of *in vitro* inhibition diameter and percent survival of the treated plants with correlation coefficient $r = 0.68959$. However, there was negative and significant ($P = 0.0113$) correlation between dry weight and AUDPC with correlation coefficient $r = -0.91198$.

3.3. Identification of the Rhizobacteria

The antagonistic isolates were identified using Gas Chromatography FAME technique. Accordingly, strain BC was identified as *Bacillus pumilus*, BS-wly as *S. marcescens*, PF9 as *Paenibacillus macerans*, PFMRI as *Bacillus subtilis*, BS-DFS as *Paenibacillus macerans*, and PF20 as *Pseudomonas fluorescens* with similarity indexes of 0.86, 0.59, 0.75, 0.71, 0.84, and 0.75, respectively (Table 4). The isolates were stored at −20 °C in ependuroff tubes containing 20% glycerol and 80% nutrient broth at the National Plant Protection Research Center (PPRC), Phytobacteriology laboratory, Ambo, Ethiopia for future research works in this area.

4. Discussion

The eight bacterial antagonists that were studied *in vitro* for their antagonistic activity against the pathogen have shown different degrees of antagonism. In the study *B. subtilis* PFMRI, *P. macerans* BS-DFS, and PF9, and *P. fluorescens* PF20 have shown the highest degree of inhibition *in vitro*. The mechanism involved during *in vitro* inhibition could be due to antibiosis, siderophore production or both (Adesina et al., 2007). This implies that the antagonists have potential to be used in the greenhouse for *in vivo* bioprotection of potato plant. The *in vitro* antagonistic activity of *B. subtilis* was also reported by Shekhawat et al. (1993) that *Bacillus* spp. isolate S4 had the greatest inhibition zone *in vitro* against *R. solanacearum* than isolate PF1 and PF2 of *P. fluorescens*. Moreover, Arsenijevic et al. (1998) reported that *Bacillus* sp. strain Fo-9 have significantly inhibited the growth of *Xanthomonas axonopodis* pv. *phaseoli* (= *X. campestris* pv. *phaseoli*) (Goszczynska et al., 2000) with the mean inhibition diameter greater than 20 mm.

There was a slight difference in *in vitro* inhibition of *R. solanacearum* by the two strains of *P. macerans* BS-DFS and PF9. However, the difference was not significant ($P > 0.05$). The *in vitro* antagonistic activity of *P. macerans* was also reported by Wakelin et al. (2002) where *P. macerans* PT1 had inhibited mycelial growth of

Table 4
Identified bacterial isolates based on Gas Chromatograph FAME technique, place of collection, their host plant and the similarity index of the strains

Strain	Bacterial species	Place of collection	Plant (rhizosphere)	Similarity index (SI)
BC	*Bacillus pumilus*	Ambo	Tomato	0.86
BS-wly	*Serratia marcescens*	Wolayta	Enset	0.59
PF9	*Paenibacillus macerans*	Ziway	Eggplant	0.75
BS-2ab	*Klebsiella pneumoniae*	Alaba	Potato	0.75
PFMRI	*Bacillus subtilis*	Harar	Potato[a]	0.71
BS-JM	*Klebsiella pneumoniae*	Jimma	Potato	0.56
BS-DFS	*Paenibacillus macerans*	Ambo-PPRC	Fallowed land[b]	0.84
PF20	*Pseudomonas fluorescens*	Ambo-Mutulu	Potato	0.75

[a] No bacterial wilt disease is reported.

[b] The formerly bacterial wilt sick plot at the National Plant Protection Research Center (PPRC), eventually failed to establish the sick plot at consistent level because of suppressive character of the soil.

Aphanomyces euteiches in dual-culture assays. Martin et al. (2003) reported that *Paenibacillus kobensis* M. was capable of inhibiting the growth of a wide range of gram positive and gram negative bacteria including several human and plant pathogens, *in vitro*. Moreover, von der Weid et al. (2003) reported that *Paenibacillus peoriae* strain NRRL BD-62 was antagonistic to broad spectrum of phytopathogenic bacteria including *R. solanacearum*. No previous report was encountered on the inhibitory activity of strains of *P. macerans* against *R. solanacearum*, in particular. This work could be the first report of *P. macerans* antagonism to *R. solanacearum*.

The inhibitory activity of *P. fluorescens* against the pathogen in the study is in line with that of Hartman et al. (1993), Shekhawat et al. (1993), Ran et al. (2005), Henok et al. (2007), and Lemessa and Zeller (2007), where they reported that isolates of *P. fluorescens* and fluorescent pseudomonads had significantly inhibited the *in vitro* growth of *R. solanacearum*. Notz et al. (2001) reported that the production of antibiotic compound 2, 4-diacetylphloroglucinol by *P. fluorescens* played a pivotal role in antagonizing plant pathogens.

Out of the six antagonistic bacterial isolates tested in the green house, *in vivo*, the highest degree of increment of plant biomass (plant height and dry weight), reduction of wilt incidence and retaining the lowest AUDPC was recorded by isolates *B. subtilis* PFMRI, *P. macerans* BS-DFS and PF9, and *P. fluorescens* PF20. Moreover, the isolates significantly suppressed the pathogen growth and perpetuation; and increased plant height and dry matter both when applied in the absence and presence of the pathogen. Lemessa and Zeller (2007) also documented similar finding where antagonistic isolates like pseudomonad APF1 and *B. subtilis* B2G significantly reduced AUDPC by 60 and 56%, respectively.

Shekhawat et al. (1993) reported that *Bacillus* sp. (S1) increased yield of potato by an average of 34% from that in the control, besides reducing wilt incidence by an average of 72%. There are some *Bacillus* spp. that affect only the biomass and do not reduce disease incidence. In this regard, Shekhawat et al. (1993) reported that the *B. subtilis* isolates (BS2 and BS3) were less effective in reducing wilt incidence in both glasshouse and field conditions. However, isolates BS2 and BS3 increased potato yield by 118 and 110%, respectively, compared to the control. This indicates the significant effect of the isolates on plant biomass, in spite of their ineffectiveness in reducing wilt incidence. Shekhawat et al. (1993) also reported that *Bacillus* sp. (S1) reduced wilt by an average of 72% compared to the control. Aspiras and Cruz (1986) also reported that *B. polymyxa* had increased the survival of tomato against bacterial wilt from 0% without antagonist to 60% with antagonist. Ryan et al. (2001) reported that *B. subtilis* GB03 and *Bacillus amyloquefaciens* IN937a were able to promote plant growth indirectly through ISR (Induced Systemic Resistance). This happens through secretion of volatiles, which in turn activate an ISR Pathway in *Arabidopsis* seedlings challenged with the soft rot pathogen *Erwinia caratovora* sub. sp. *caratovora*. The same mechanism may have been involved to induce ISR in potato against *R. solanacearum* by *B. subtilis* PFMRI. One can extrapolate that the *B. subtlis* strains like PFMRI with the ability to reduce wilt incidence and increase plant biomass simultaneously may have great potential to be used in the management options against bacterial wilt if also equally effective under field conditions.

Gardener McSpaden, 2004 reported that nitrogen fixation occurs in *Paenibacillus azotofixans*, *P. macerans*, and *P. polymyxa*, but does not seem to occur in *Bacillus* sp. This could be one of the major reasons for the highest relative effect of *P. macerans* DFS and PF9 on plant biomass as compared to the rest of antagonistic bacterial strains studied. Wakelin et al. (2002) reported that *P. macerans* PT1 had increased the root weight of pea plant under glasshouse conditions even in the presence of the pathogen.

Pseudomonas fluorescens PF20 significantly increased the plant height and dry weight of potato next to *B. subtilis* PFMRI. Mechanism of action involved during biomass increment could be the same as the one reported by Duijff et al. (1997) that after inoculation of tomato with endophytic *P. fluorescens* WCS 417r, a thickening of the outer tangential and outermost part of the radial side of the first layer of cortical cell walls occurred when epidermal or hypodermal cells were colonized. This will result in increasing plant biomass in addition to creating a barrier to the pathogen entrance. It was also suggested by Gray and Smith (2005) that these endophytic bacteria may be formerly rhizosphere inhabitants which penetrate in to the plant body/system, thereby establishing a symbiotic association with the host plant. In this study bacterial antagonists were initially applied to potato eyepieces, thus, the antagonists may have penetrated the plant through root rhizosphere and established an endophytic life style enabling the plant bioprotection from the pathogen. The beneficial attributes of strains of *P. fluorescens* are their direct inhibitory effect on the pathogen, ability to grow rapidly and to colonise potato root systems and ability to enhance growth of potato plants (Kloepper et al., 1980; Aspiras and Cruz, 1986).

Bacillus subtilis PFMRI and *P. macerans* BS-DFS and PF9 were the top performing bacterial antagonists. The Harar (eastern part of Ethiopia) isolate *B. subtilis* PFMRI is the bacterium, which was originally isolated from a potato field where there was no report of bacterial wilt disease. Moreover, the antagonist was also effective in suppressing the growth and perpetuation of the pathogen both *in vitro* and *in vivo* in this study. Thus, the absence of disease in the field from which the antagonist was originally isolated could be due to suppressive effect of the bacterial bioagents like *B. subtilis* PFMRI. By the same token, strain BS-DFS of *P. macerans* was isolated from the National Plant Protection Research Centre (PPRC), Ambo, Ethiopia experimental field where the pathogen was introduced to develop Bacterial Wilt (*R. solanacearum*) Sick Plot, but not established. This isolate was also one of the top antagonists of the pathogen both *in vitro* and in the greenhouse, *in vivo*. The presence of potential bacterial antagonists like strain BS-DFS of *P. macerans* may have played a pivotal role in suppressing the disease establishment despite high artificial inoculation of the soil with the pathogen. This indicates the potential of the antagonist to suppress disease establishment, even under conditions of high pathogen inocula. In addition, strains BS-DFS and PF9 of *P. macerans* were isolated from different geographical locations of the country (Ethiopia) but have shown overlapping performance during both *in vitro* and *in vivo* studies. However, further evaluation of the effects of multiple antagonistic strains collected from different parts of the country on disease reduction and growth promotion need to be conducted to substantiate these findings. Furthermore, the antagonistic strains of *B. subtilis* and *P. macerans* have shown relative consistency both *in vitro* and *in vivo*, in significantly suppressing the pathogen's growth and perpetuation. However, the majority of the antagonistic strains, which failed to repeat the strong *in vitro* inhibition that they have shown in the laboratory in the *in vivo* bioprotection in the greenhouse, did not share such consistency. Ran et al. (2005) and Lemessa and Zeller (2007) reported lack of consistency in *in vitro* antagonism and *in vivo* biological control. Thus, it can be concluded that the use of antagonistic isolates like *B. subtilis* PFMRI and *P. macerans* BS-DFS and PF9 have potential in potato bioprotection or as part of an integrated disease management package for bacterial wilt management. However, additional testing is still needed to prove efficacy under a variety of field conditions.

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References

Adesina, M.F., Lembke, A., Costa, R., Speksnijder, A., Smalla, K., 2007. Screening of bacterial isolates from various European soils for *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: site-dependent composition and diversity revealed. Soil Biology and Biochemistry 39, 2818–2828.

Arsenijevic, M., Obradovic, A., Stevanovic, D., Ivanovic, M., 1998. Antagonistic effect of some saprophytic bacteria to *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli*. Biological Control of Fungal and Bacterial Plant Pathogens, IOBC Bulletin 21 (9), 297–300.

Aspiras, R.B., Cruz, A.R., 1986. Potential biological control of bacterial wilt in tomato and potato with *Bacillus polymyxa* FU6 and *Pseudomonas fluorescens*. In: Parsley, G.J. (Ed.), Bacterial wilt disease in Asia and the South Pacific, vol. 13. ACIAR Proceedings, pp. 89–92.

Bashan, Y., Holguin, G., 1998. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPR (plant growth-promoting bacteria) and PGPR. Soil Biology and Biochemistry 30, 1225–1228.

Campbell, C.L., Madden, L.V., 1990. Introduction to Plant Disease Epidemiology. Wiley, NY.

CIP (International Potato Centre), 1995. Program report: 1993–1994. Lima, Peru. 192p.

Compant, S., Duffy, B., Nowak, J., Clement, C., Barka, E.A., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied and Environmental Microbiology 71, 4951–4959.

Cook, D., Barlow, E., Sequeira, L., 1989. Genetic diversity of *Pesudomonas solanacearum*: detection of restriction fragment length polymorphisms with DNA probes that specify virulence and the hypersensitive response. Molecular Plant-Microbe Interactions 2, 113–121.

Dhingra, Onkar D., Sinclair, James B. (Eds.), 1995. Basic Plant Pathology Methods, 2nd ed. CRC Press, USA.

Duijff, B.J., Gianinazzi-Pearson, V., Lemanceau, P., 1997. Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. New Phytologist 135, 325–334.

Elphinstone, J.G., Aley, P., 1993. Integrated control of bacterial wilt of potato in the warm tropics of Peru. In: Hartman, G.L., Hayward, A.C. (Eds.), Bacterial Wilt. Proceedings of an International Conference held at Kaohsiung, 28–31 October 1992, Taiwan. ACIAR Proceedings, vol. 45, pp. 276–283.

Gardener McSpaden, B.B., 2004. Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. Phytopathology 94, 1252–1258.

Garrett, K.A., Mundt, C.C., 2000. Host diversity can reduce potato late blight severity for focal and general patterns of primary inoculum. Phytopathology 90, 1307–1312.

Goszczynska, T., Sefontein, J.J., Serfontein, S., 2000. Introduction to Practical Phytobacteriology. ARC-Plant Protection Research Institute, Pretoria, South Africa.

Gray, E.J., Smith, D.L., 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. Soil Biology and Biochemistry 37, 395–412.

Hallman, J., Quadt-Hallman, A., Mahafee, W.F., Kloepper, J.W., 1997. Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology 43, 895–914.

Hartman, G.L., Hong, W.F., Hanudin, Hayward, A.C., 1993. Potential of biological and chemical control of bacterial wilt. In: Hartman, G.L., Hayward, A.C. (Eds.), Bacterial Wilt. Proceedings of an International Conference held at Kaohsiung, 28–31 October 1992, Taiwan (EIAR) ACIAR Proceedings, pp. 322–326.

Henok, K., Fassil, A., Yaynu, H., 2007. Evaluation of of *Pseudomonas fluorescens* isolates as biocontrol agents against bacterial wilt caused by *Ralstonia* (*Pseudomonas*) *solanacearum*. Pest Management Journal of Ethiopia 11, 9–18.

Kloepper, J.W., Rodriguez-Ubana, R., Zehnder, G.W., Murphy, J.F., Sikora, E., Fernandez, C., 1999. Plant root-bacterial interactions in biological control of soilborne diseases and potential extension to systemic and foliar diseases. Australasian Plant Pathology 28, 21–26.

Kloepper, J.W., Schroth, M.N., Miller, T.D., 1980. Effects of rhizosphere colonisation by plant growth promoting rhizobacteria on potato plant development and yield. Phytopathology 2, 1078–1082.

Lemessa, F., Zeller, W., 2007. Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia. Biological Control 42, 336–344.

Martin, Nathaniel I., Haijing, Hu, Moake, Matthew M., Churey, John J., Whittal, Randy, Worobo, Randy W., Vederas, John C., 2003. Isolation, structural characterization, and properties of Mattacin (Polymyxin M), a cyclic peptide antibiotic produced by *Paenibacillus kobensis* M. Journal of Biological Chemistry 278 (15), 13124–13132.

MoARD (Ministry of Agriculture and Rural Development), 2005. Crop variety register. Crop Development Department, Issue No. 8. April 2005, Addis Ababa Ethiopia. 170pp.

Naser, A., 2008. Study of potato (*Solanum tuberosum*) cultivars for their reaction to *Ralstonia solanacearum* and the relationship of latent infection with field wilt incidence in Ethiopia. International Journal of Pest Management, (Under Review).

Naser, A., Tesfaye, B., 2005. Search for potential bacterial antagonists for the control of potato bacterial wilt pathogen (*Ralstonia solanacearum*). In: Ethiopian Institute of Agricultural Research (EIAR), Plant Protection Research Centre Progress Report for the Period 2004/05, pp 78–82.

Naser, A., Chemeda, F., Yaynu, H., 2007. Phenotypic characterization of isolates of *Ralstonia solanacearum* and evaluation of some tomato varieties against bacterial wilt. Pest Management Journal of Ethiopia 11, 45–59.

Nganga, S., 1982. Potato development and transfer of technology in tropical Africa. A Regional Symposium. A Regional Symposium. CIP, ILCA, Addis Ababa, Ethiopia.

Notz, R., Maurhofer, M., Schnider-keel, U., Duffu, B., Haas, D., Defago, G., 2001. Biotic factors affecting expression of the 2, 4-diacetyl phloroglucinol biosynthesis gene *phlA* in *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. Phytopathology 91, 873–881.

Ran, L.X., Liu, C.Y., Wu, G.J., van Loon, L.C., Bakker, P.A.H.M., 2005. Suppression of bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. in China. Biological Control 32, 111–120.

Ryan, P.R., Delhaize, E., Jones, D.L., 2001. Function and mechanism of organic anion exudation from plant roots. Annual Review of Plant Physiology and Plant Molecular Biology 52, 527–560.

SAS Institute Inc., 1999. The SAS® System FOR WINDOWS™. Version 8, Cary, NC, USA.

Sasser, M., 2001. Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note 101.

Shekhawat, G.S., Chakrabarti, S.K., Kishore, V., Sunaina, V., Gadewar, A.V., 1993. Possibilities of Biological Management of Potato Bacterial Wilt with Strains of *Bacillus* spp., *B. subtilis*, *Pseudomonas fluorescens* and *Actinomycetes*. In: Hartman, G.L., Hayward, A.C. (Eds.), Bacterial Wilt. Proceedings of an International Conference held at Kaohsiung, 28–31 October 1992, Taiwan, vol. 45. ACIAR Proceedings, pp. 327–328.

Sturz, A.V., Christie, B.R., Nowak, J., 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. Critical Reviews in Plant Sciences 19, 1–30.

Van Loon, L.C., Bakker, P.A.H.M., Pieterse, J.C.M., 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36, 453–483.

von der Weid, I., Alviano, D.S., Santos, A.L.S., Soares, R.M.A., Alviano, C.S., Seldin, L., 2003. Antimicrobial activity of *Paenibacillus peoriae* strain NRRL BD-62 against a broad spectrum of phytopathogenic bacteria and fungi. Journal of Applied Microbiology 95, 1143–1151.

Wakelin, S.A., Walter, M., Jaspers, M., Stewart, A., 2002. Biological control of *Aphanomyces euteiches* root-rot of pea with spore-forming bacteria. Australasian Plant Pathology 31, 401–407.

Yaynu, H., 1989. Characteristics of isolates of *Pseudomonas solanacearum* in Ethiopia. Ethiopian Journal of Agricultural Science 11, 7–13.