Available online at www.sciencedirect.com
ScienceDirect
Biological Control 42 (2007) 336–344
Biological Control
www.elsevier.com/locate/ybcon

Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia

F. Lemessa *, W. Zeller

Institute for Biological Control, Federal Biological Research Centre for Agriculture and Forestry (BBA), Heinrichstr. 243, 64287 Darmstadt, Germany

Received 23 February 2007; accepted 21 May 2007
Available online 29 May 2007

Abstract

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) has become a severe problem mainly on potato and tomato in Ethiopia and no effective control measure is available yet. To explore possibilities for the development of biological control for the disease, 118 rhizobacteria, most of them collected from Ethiopia, were screened against an Ethiopian *R. solanacearum* strain. On the basis of *in vitro* screening, six strains (RP87, B2G, APF1, APF2, APF3, and APF4) with good inhibitory effect were selected for *in planta* testing in a greenhouse. In the greenhouse, soil and tomato seedlings were treated with the antagonists and their effects studied. The study showed that APF1 and B2G strains significantly reduced disease incidence and increased weight of tomato plants. Area under disease progress curves (AUDPC) was reduced by 60% and 56% in plants inoculated with APF1 and B2G strains, respectively. Plant dry weight increase in plants inoculated with APF1 and B2G strains was 96% and 75%, respectively. APF1 was found to be the most beneficial strain in disease suppression and also growth promotion resulting in 63% dry weight increase compared to untreated control. The study revealed that APF1 and B2G strains are promising strains whose effectiveness under field conditions and their mode of action should be investigated.

Keywords: Rhizobacteria; Biocontrol; Bacterial wilt; *Ralstonia solanacearum*; *Bacillus subtilis*; *Streptomyces setonii*; Fluorescent pseudomonads

1. Introduction

Ralstonia solanacearum (Smith) (Yabuuchi et al., 1995) is an important soilborne bacterial plant pathogen with a worldwide distribution and a wide host range of more than 200 species in 50 families (Hayward, 1991). Some of its economically important plant hosts include tomato, potato, eggplant, pepper, tobacco, banana, chilli, and peanut (French and Sequeira, 1970). In Ethiopia *R. solanacearum* is an important disease of potato and tomato (Yaynu, 1989) and its importance is increasing from time to time.

To date, no effective control method has been developed for this wilt disease. Plant breeding, field sanitation, crop rotation, and use of bactericides have met with only limited success (Ciampi-Panno et al., 1989). Although disease resistance is an important component of integrated disease management, it is generally agreed that breeding for resistance is not completely effective, producing only modest gains and often lacking stability and/or durability (Hayward, 1991; Boucher et al., 1992). Furthermore, the high variability of strains of *R. solanacearum* (Elphinstone, 1992) combined with the influence of environmental factors on host–pathogen interactions (Hayward, 1991) often restricts the expression of resistance to specific regions.

Various recent studies have indicated that biological control of bacterial wilt disease could be achieved using antagonistic bacteria (McLaughlin et al., 1990; Ciampi-Panno et al., 1989). Toyota and Kimura (2000) have reported the suppressive effect of some antagonistic bacteria on *R. solanacearum*. Moreover, Ciampi-Panno et al. (1989) has proved the use of antagonistic pathogens to be effective in control

* Corresponding author. Present address: Department of Crop Sciences, College of Agriculture and Veterinary Medicine, Jimma University, P.O. Box 307, Jimma, Ethiopia. Fax: +251 471110934.
E-mail address: lemessaf@yahoo.com (F. Lemessa).

1049-9644/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2007.05.014

of *R. solanacearum* under field condition. Potential biological agents used to control bacterial wilt caused by *R. solanacearum* include avirulent mutants of *R. solanacearum* (Dong et al., 1999), genetically engineered antagonistic bacteria (Kang et al., 1995), and some naturally occurring antagonistic rhizobacteria such as *Bacillus* spp. (Silveira et al., 1995), *Pseudomonas* spp. (Guo et al., 2001), and *Streptomyces* spp. (El Albyad et al., 1996). However, current interest in the possible release of genetically modified microorganisms into the environment has raised concerns over issues of environmental health. Thus naturally occurring microorganisms remain the potential candidates.

Soilborne diseases have been controlled more recently by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants (Thomshaw, 1996). The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large and metabolically active groups of bacteria (Villacieros et al., 2003) known as plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980). PGPR are known to rapidly colonize the rhizosphere and suppress deleterious microorganisms as well as soilborne pathogens at the root surface (Rangajaran et al., 2003). These organism can also be beneficial to the plant by stimulating growth (Bloemberg and Lugtenberg, 2001).

Therefore, the aim of this study was to isolate rhizobacteria and screen them for *in vitro* and *in planta* antagonistic activity against *R. solanacearum* for biological control in Ethiopia.

2. Materials and methods

2.1. Isolation of potential antagonistic bacteria

A total of 98 bacteria were collected from rhizosphere of potato, tomato, pepper, coffee, and maize plants from Jimma and its surroundings in Ethiopia during March to May 2005. For isolation from the rhizosphere, plant roots were gently washed twice in sterile water to remove adhering soil, and then root sections of approximately 1 g were added to 200 ml sterile water in flasks and shaken on rotary shaker at 150 rpm for 30 min. Then serial dilutions of the root suspension were plated on King's medium B agar (KB) (King et al., 1954) and Luria-Bertani agar (LB) (Sambrook et al., 1989) and incubated at 28 °C for 48 h. When the bacterial colony appeared on the medium, representative isolates were picked for antagonism study. In addition to the rhozospheric bacteria isolated from Ethiopia, 20 species of bacterial strains, which were isolated mainly from the rhizosphere of potato, were procured from Rostock University, Germany, and included in the screening study. For long-term preservation, bacteria were stored in 20% glycerol at −70 °C.

2.2. Bacterial strain and culture conditions

As the majority of Ethiopian *R. solanacearum* strains were identified as biovar II race three groups in our previous study (Lemessa and Zeller, 2007), a representative strain IBC Pot 4JU was used in the screening study. The strain was originally isolated from wilted potato from Jimma, Ethiopia, and identified as *R. solanacearum* by tomato bioassay and species specific PCR primers (759/760). IBC Pot 4JU belongs to biovar II as determined according to Hayward (1964) and race 3 according to the standard set of Buddenhagen et al. (1962). It is pathogenic to potato, tomato, and eggplant but not to pepper and tobacco (Lemessa and Zeller, 2007). The strain was routinely cultured on casamino acids peptone glucose (CPG) agar (Smith et al., 1995) and on tetrazolium chloride (TTC) agar (Kelman, 1954) at 28 °C for 48 h and temporarily stored in sterile water at room condition.

2.3. In vitro antagonistic activity

In vitro antagonism studies between rhizospheric bacteria and the pathogenic strain of *R. solanacearum* IBC Pot 4JU were carried out on KB and LB agar plates using chloroform vapour (Ryan et al., 2004) and agar diffusion methods (Mitchell and Carter, 2000).

2.3.1. Chloroform vapour method

Candidate antagonistic bacteria were spotted on KB and LB media and incubated for 48 h at 28 °C. After 48 h of incubation, the growing antagonists were killed by inverting Petri dishes over chloroform for 3 min. The test strain was cultured in CPG broth on rotary shaker for 24 h and centrifuged at 10,000 rpm and cell pellets were diluted in 0.85% (w/v) NaCl solution and adjusted to 10^8 cfu/ml. The plates in which antagonists were grown were flooded with 2 ml cell suspension of the pathogen, dried, and incubated for two or more days. The effectiveness of strains was evaluated by measuring the inhibition zones around antagonistic bacteria. The experiment was performed with a completely randomized design with three replications and repeated twice.

2.3.2. Agar-diffusion test

One-hundred microliters of *R. solanacearum* suspension containing 10^8 cfu/ml was spread on KB and LB plates and four holes of 9 mm diameter punched into the agar. In these holes 30 µl suspension of each test antagonist ($\approx 10^9$ cfu/ml) was added and the plates incubated at 28 °C for 48 h. Inhibition of *R. solanacearum* growth was assessed by measuring the radius of inhibition zone (mm) after incubation for 48 h at 28 °C.

2.4. Greenhouse evaluation of rhizobacterial isolates

2.4.1. Growth of plants

A potting medium that constitutes a mixture (1:3) of sand: commercial potting substrate (FRUHSTORFER ERDE Typ LD 80; Industrie-Erdenwerk Archut, Lauterbach, Germany) was sterilized at 121 °C for 20 min and filled in a sterilized plastic tray. Tomato c.v 'Matina' seed

was obtained from the HILD Samen GmbH, Marbach, Germany. Seeds were surface sterilized with 2% sodium hypochlorite for 2 min (Guo et al., 2004), washed thoroughly with sterilized water and planted in plastic tray filled with the sterilized potting medium. The plants were maintained in a greenhouse at temperatures of 24–28 °C and 75–90% relative humidity and seedlings were watered with sterile water when necessary.

2.4.2. Bioassay

Six strains of bacteria with the greatest inhibition in *in vitro* test were further tested in greenhouse on tomato plants to evaluate their ability to control bacterial wilt *in planta*. For this purpose, the same potting mix used for raising tomato seedlings above was used after autoclaving. The pathogen was prepared by culturing in CPG broth for 48 h at 28 °C and 150 rpm on rotary shaker. Cultures were centrifuged at 10,000 rpm (Beckman J2-21 M/E centrifuge, USA) for 10 min at 10 °C. Bacterial pellets were suspended in distilled water and adjusted to 10^8 cfu/ml. Four-hundred grams of the sterilized potting medium was mixed with 75 ml of *R. solanacearum* IBC Pot 4JU at 10^8 cfu/ml ($\approx 1.2 \times 10^6$ cfu/g soil, dry weight) and placed in 12 cm diameter pots. One week after incorporation of the pathogen into the soil and one day before transplanting the test plants, antagonists were incorporated in the soil at a rate of 50 ml per pot (400 g) at 10^9 cfu/ml. On the next day, four weeks old tomato seedlings raised in plastic tray were root dipped in suspension (10^9 cfu/ml) of antagonistic bacteria for 60 min and transplanted into pathogen–antagonist mixture soil. Plants were kept in the greenhouse at 24–28 °C and 75–90% relative humidity in 12 h light and 12 h dark conditions. The experiment was conducted two times with completely randomised design. Treatments were replicated five times with 12 plants per replication.

2.4.3. Disease assessment

The percentage of diseased plants was scored separately per pot at different time points based on wilting symptoms and the area under disease progress curves (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 per cent and time (t) in days, AUDPC was expressed in %-days (Campbell and Madden, 1990). Biological control efficacy was calculated according to Guo et al. (2004) as:

$$\mathrm{BCE} = [(\mathrm{D_C} - \mathrm{D_T})/\mathrm{D_C}] \times 100\%,$$

where $\mathrm{D_C}$ is disease of control and $\mathrm{D_T}$ is disease of the treatment group.

2.4.4. Population dynamics of R. solanacearum in soil treated with antagonists

To assess the effect of antagonists on the population density of *R. solanacearum* in soil, 2 g of pathogen–antagonist infested soil samples were taken from each pot of treatments at different intervals (0, 3, 6, and 9 weeks after treatment) giving 12 g of soil per treatment. The soil was mixed thoroughly, and then 1 g was added to sterile distilled water (1:9, wt/vol) and shaken for 30 min on a rotary shaker, serial dilutions were made, and 0.1 ml aliquots were spread on the surface of a semi-selective SMSA medium (Englerbrecht, 1994). The medium is made of: 10 g bacto peptone (Difco), 5 ml glycerol, 1 g casamino acid (Difco), 15 g bacto agar (Difco), and 1 L distilled water. This medium was supplemented with 1% polymyxin B sulphate (Sigma), 1% crystal violet, 1% tetrazolium salt (Sigma), 1% bacitracin (Sigma), 0.1% penicillin (Sigma), 1% chloramphenol (Sigma), and 1% cycloheximide (Sigma). After incubating plates at 28 °C for 3 days, colonies of *R. solanacearum* were counted and cfu were calculated per gram (dry weight) of potting medium. Four replicates were prepared for each sample. Since populations of bacteria approximate a log normal distribution (Loper et al., 1984), values were log transformed before analysis to normalize variance.

2.4.5. Plant weight and growth promotion assessment

At the end of the experiment (2 months after transplanting), plants including the roots were harvested from the pots and fresh weight recorded. Healthy plants were counted and uprooted separately and their weights recorded to measure growth promotion, compared with the untreated control (Lim and Kim, 1997). For dry weight measurement, plants were dried in an oven at 60 °C for 3 days and weights evaluated for each treatment. Dry and fresh weights were used for data analysis.

2.4.6. Statistical analysis

The data were subjected to analysis of variance using SAS version 8 (SAS Institute, 1999). Single and interaction effects of factors were determined using the GLM procedure of SAS. Correlation analysis was carried out using the CORR procedure. Whenever significant interactions were observed between factors, the level of one factor was compared at each level of the other factor. Mean values among treatments were compared by the Tukey's test at $\alpha = 0.05$ level of significance.

2.5. Identification of the bacterial isolates

Six bacterial strains with the highest inhibitory effect *in vitro* were selected and used during the study. Two of them were *Streptomyces setonii* RP87 (Millard and Burr) and *Bacillus subtilis* B2G (Ehrenberg) that were obtained

from Rostock University, Germany, and included in our study. The remaining four were strains from the collection from Ethiopia and taxonomically differentiated on the basis of their reactions to standard biochemical tests from Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984): pigmentation on KB medium, Gram stain, oxidase and catalase test, starch hydrolysis, gelatine liquefaction, and growth on carbon sources (fructose, sucrose, glucose, and galactose).

3. Results

3.1. In vitro assays

A total of 118 rhizospheric bacteria were screened against *R. solanacearum* strain IBC Pot 4JU and 23 strains had inhibitory effect that ranged from an average of 0.5–11 mm radius of inhibition zone. Among those 23 strains, 16 (70%) were fluorescent under UV light when cultured on KB medium. Six strains with inhibition zone of 5–11 mm radius were selected and used for further study. Two of the strains were *B. subtilis* B2G and *Streptomyces setoni* RP87 which were from Rostock University, Germany, and four (APF1, APF2, APF3, and APF4) were from the collection of Ethiopia; all were identified as fluorescent pseudomonads based on the Bergey's Manual. The four Ethiopian strains are currently maintained in the laboratory of Plant Pathology at College of Agriculture and Veterinary Medicine, Jimma University, Ethiopia.

The results of *in vitro* tests indicated significant differences among antagonistic strains and between media (LB and KB) used (Table 1). Methods of *in vitro* test (chloroform vapour and agar diffusion) did not significantly affect growth inhibition. The two-way interaction, medium by antagonist, was significant ($P < 0.0001$), indicating that the strains differed in inhibiting the pathogen depending on type of medium. On LB medium, strains RP87 and B2G exhibited significantly greater inhibition zones than the other four strains, while on KB significantly greater inhibition was recorded by RP87, B2G, APF1, and APF2 (Table 1). APF1 and APF2 exhibited significantly greater inhibition on KB than on LB. Inhibitory effect of strains RP87, B2G, APF3, and APF4 was not significantly affected by type of media.

Table 1
In vitro inhibition (mm) of growth of *Ralstonia solanacearum* strain by antagonistic bacterial isolates (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and fluorescent pseudomonads APF1, APF2, APF3, and APF4) in chloroform vapour and agar diffusion methods on Luria-Bertani (LB) and King's B (KB) media incubated at 28 °C for 48 h in laboratory

Antagonistic strains	Chloroform vapour		Agar diffusion	
	LB	KB	LB	KB
RP87	10.7 a A	10.3 a A	10.0 a A	10.2 a A
B2G	10.3 a A	10.0 a A	10.3 a A	10.1 a A
APF1	6.0 b B	12.1 a A	6.0 b B	12.3 a A
APF2	5.3 b B	13.0 a A	4.0 b B	12.0 a A
APF3	4.1 b B	6.0 b B	4.2 b B	6.7 b B
APF4	3.7 b B	5.3 b B	3.7 b B	5.7 b B

Means within column (row) followed by the same lower (upper) case letters are not significantly different (Tukey, $\alpha = 0.05$).

3.2. Greenhouse experiment

The *in planta* efficacy of selected antagonists for the control of *R. solanacearum* wilt in tomato plants was evaluated under greenhouse conditions and there were no significant differences until 45 days after transplanting (Fig. 1). At 45 days after transplanting, *B. subtilis* B2G and the pseudomonad APF1 and APF2 strains significantly reduced wilt incidence ($P < 0.0001$) compared to the control. At 57 days after transplanting, only *B. subtilis* B2G and pseudomonad APF1 strains significantly reduced disease incidence ($P = 0.0007$) compared to the control. *Streptomyces setonii* RP87 and the pseudomonad APF2 and APF3 did not significantly differ from the control throughout the experiment. Generally, the pseudomonad APF1 and *B. subtilis* B2G strains reduced disease incidence at 45 days after transplanting by 70% and 63% and at 57 days after transplanting by 53% and 52%, respectively.

Wilt incidence in the form of AUDPC was also significantly affected by treatments. Plants treated with *B. subtilis* B2G, and pseudomonad APF1 and APF2 strains sustained significantly lower AUDPC ($P = 0.0022$) compared to control (Table 2). Though the strain *S. setonii* RP87 showed very high inhibition in *in vitro* test, it failed to reduce disease in the *in vivo* biocontrol assay. Generally, poor

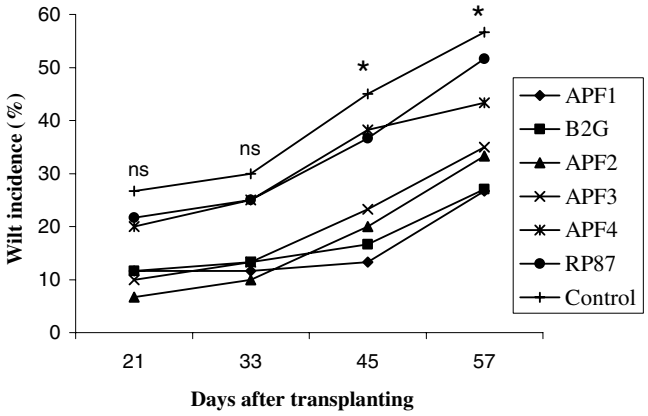

Fig. 1. Bacterial wilt symptom development expressed as wilt incidence on tomato plants treated by different bacterial antagonists (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and fluorescent pseudomonads APF1, APF2, APF3, and APF4). The root system of 4-week-old plants were dipped in 10^9 cfu/ml antagonistic bacterial suspensions or sterile water (control) for 60 min, then transplanted into autoclaved soil artificially infested with the pathogen (10^8 cfu/ml) and bacterial antagonists (10^9 cfu/ml) and grown in the greenhouse at 25–28 °C for 60 days. Incidence of the disease was calculated by counting the number of plants showing typical diseases symptoms at 21, 33, 45, and 57 days after transplanting. Control plants grown in non-infested soil were free of symptoms and not included in statistical analysis. ns and * indicate absence and presence of significant differences (Tukey's test, $\alpha = 0.05$) among treatments at a particular day after transplanting, respectively.

correlation (data not shown) was found between *in vitro* antagonism and *in planta* suppression of disease. Biocontrol efficacy as estimated by AUDPC ranged from 12.6% to 60.3% and the highest biocontrol efficacy was recorded by pseudomonad APF1 followed by *B. subtilis* B2G and pseudomonad APF2 (Table 2).

Population densities of *R. solanacearum* (log cfu/g, dry weight) in the soil after incorporation of antagonists into potting medium is shown in Fig. 2. Up to 3 weeks after incorporation of antagonists into soil, population density of *R. solanacearum* declined in all treatments including the control. Later, populations of the pathogen started to increase, however, differently among the treatments. Six weeks after soil treatment, population of the pathogen with pseudomonad APF1 and *B. subtilis* B2G were significantly ($P = 0.0018$) lower than the control. However, 9 weeks after soil treatment population of the pathogen was significantly ($P = 0.0002$) lower only in soil treated with pseudomonad APF1 strain. Generally, per cent reductions of *R. solanacearum* population with pseudomonad APF1 and *B. subtilis* B2G strains were 44% and 45%, respectively, 6 weeks post-treatment and 35% and 26%, respectively, at 9 weeks.

There were significant differences among treatments for both dry and fresh weight of tomato plants (Fig. 3a and b). Average dry weight of tomato was significantly higher ($P < 0.0001$) for plants treated with pseudomonad APF1 and *B. subtilis* B2G and average fresh weight was significantly higher ($P < 0.001$) for plants treated with strains *B. subtilis* B2G and pseudomonad APF1 and APF2. Highest dry and fresh weight increase was recorded with pseudomonad APF1 treatment: 96% and 81%, respectively. Dry and fresh weight for plants treated with *B. subtilis* B2G were 75% and 54% higher than the control, respectively. No significant difference was observed between con-

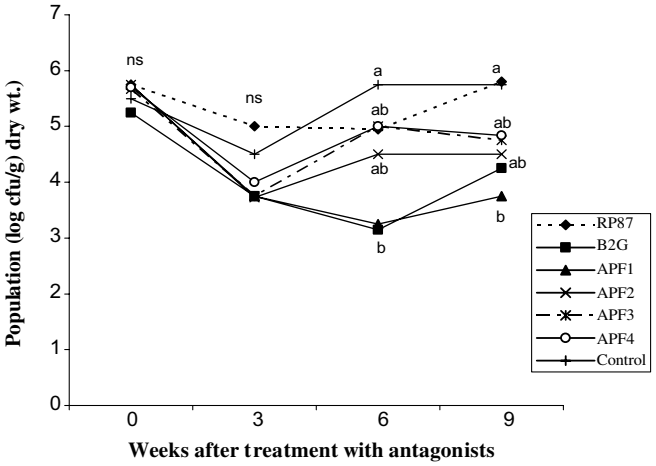

Fig. 2. Changes in population density of *Ralstonia solanacearum* in the rhizosphere of tomato at different intervals after introduction of bacterial antagonists (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and fluorescent pseudomonads APF1, APF2, APF3, and APF4) into the potting medium under greenhouse conditions at 25–28 °C. Potting medium treated only with the pathogen served as control. Means with the same letter are not significantly different (Tukey's test, $\alpha = 0.05$). ns, no significant differences among treatments.

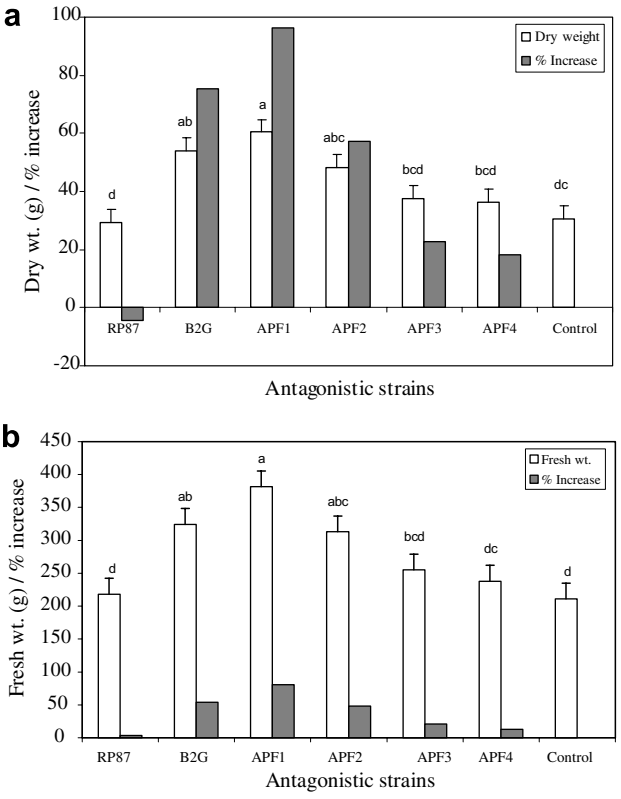

Fig. 3. Effect of antagonistic bacterial strains (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and fluorescent pseudomonads APF1, APF2, APF3, and APF4) on dry (a) and fresh (b) weight of tomato plants and per cent increase compared to control under greenhouse conditions at 25–28 °C. Dry weight was recorded after plants were dried in an oven at 60 °C for 3 days. Data are the mean of five replicates with 12 plants per replications. Bars with the same letter are not significantly different according to Tukey's test ($\alpha = 0.05$).

Table 2
Effect of antagonistic bacterial strains (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and fluorescent pseudomonads APF1, APF2, APF3, and APF4), on *Ralstonia solanacearum* wilt incidence expressed as area under disease progress curves (AUDPC) in tomato plant under greenhouse conditions

Strain[a]	AUDPC (%-days)	Biocontrol efficacy (%)
RP87	983.3 ab	12.6
B2G	493.6 b	55.6
APF1	441.6 b	60.3
APF2	499.9 b	50.0
APF3	632.6 ab	43.1
APF4	949.9 ab	14.5
Control	1111.6 a	—

[a] The root system of 4-week-old plants were dipped in 10^9 cfu/ml antagonistic bacterial suspensions or sterile water (control) for 60 min, then transplanted into autoclaved soil artificially infested with the pathogen (10^8 cfu/ml) and bacterial antagonists (10^9 cfu/ml) and grown in the greenhouse at 25–28 °C for 60 days. Control plants grown in non-infested soil were free of symptoms and not included in statistical analysis. Means in column followed by the same letter are not significantly different (Tukey's test, $\alpha = 0.05$).

trol and *S. setonii* RP87 and pseudomonad APF3 and APF4 strains in dry and fresh weights. Simple correlation analysis showed that there were negative and significant associations between disease AUDPC and tomato dry and fresh weights. AUDPC was associated with fresh and dry tomato weights with correlation coefficients $r = -0.57$ ($P < 0.0001$) and $r = -0.34$ ($P = 0.0039$) ($n = 70$), respectively.

The plant growth promotion efficiency of antagonistic isolates monitored by measuring plant biomass (fresh and dry weight) showed variation among plants treated with antagonists and the untreated control. There were significantly higher dry ($P = 0.0185$) and fresh ($P = 0.0273$) weights of tomato plants treated by pseudomonad APF1 compared with the untreated control (Table 3). However, there was no significant difference between control and other antagonistic strains. Treatment with pseudomonad APF1 resulted in 63% and 72% increases in dry and fresh weights of tomato, respectively, compared to the untreated control.

3.3. Identification of bacterial strains

For the antagonism study, six strains were used. Two of them were identified species *S. setonii* RP87 and *B. subtilis* B2G. Identification of the remaining four strains (APF1, APF2, APF3, and APF4) was carried out and all were identified as fluorescent pseudomonads on the basis of standard biochemical methods. They were Gram positive, fluorescent under UV light, and oxidase, catalase, glucose, fructose, sucrose, and galactose positive. Moreover, they liquefy gelatine but do not hydrolysis starch. Originally, RP87, B2G, APF1, and APF3 strains were isolated from potato, APF2 from tomato, and APF4 from pepper rhizospheres.

4. Discussion

Root associated bacteria are an important functional group of beneficial bacteria used for control of soilborne pathogens and plant growth promotion (Gamalero et al., 2003; Rajkumar et al., 2005). In this investigation, root associated bacteria were isolated from the rhizospheres mainly of potato, tomato, and pepper with the objective of selecting efficient antagonists against soilborne infection with *R. solanacearum*, the causal agent of bacterial wilt.

In *in vitro* screening on LB and KB media, six strains (*S. setonii* RP87, *B. subtilis* B2G, and fluorescent pseudomonads APF1, APF2, APF3, and APF4) with the highest inhibitory effect were selected for further greenhouse conditions. The *in vitro* test showed that the type of media used affects expression of inhibition by these antagonists. Accordingly, the antagonistic strains *S. setonii* RP87, *B. subtilis* B2G, and fluorescent pseudomonad APF3 and APF4 showed similar results on both LB and KB media, while the fluorescent pseudomonad APF1 and APF2 strains produced significantly higher inhibition in KB as compared to LB (Table 1). This indicated that the mechanism of inhibition of pseudomonad APF1 and APF2 strains was affected by the constituents of the media. It is known that LB is an iron rich medium (Hassett et al., 1995), while KB is a medium deficient of iron (Lim and Kim, 1997). Thus the main increase in inhibitory activity of pseudomonad strains APF1 and APF2 in KB medium could be siderophore production that inhibited *R. solanacearum* growth. This conclusion is supported by the observation that amendment of KB medium with iron reduced the inhibitory activity of pseudomonad strains APF1 and APF2 (data not shown). This is in agreement with other observations that inhibition of growth on KB could be a consequence of production of siderophores by *Pseudomonas* strains (Lim and Kim, 1997; Blanco et al., 2004). The type of culture medium strongly affects activity of antagonists by mediating production of substances that are responsible for inhibition (Montesinos et al., 1996; Chen et al., 2003). For instance, Duffy and Défago (1999) noticed that addition of zinc increases antibiotic synthesis in pseudomonads. Also the yields of exotoxin A in *P. aeruginosa* cultures were influenced by the concentration of iron in the culture medium (Bjorn et al., 1978). When the iron concentration of the culture media was increased from 0.05 to 1.5 μg/ml, there was at least a 90% decrease in exotoxin A.

Table 3
Effect of antagonistic bacterial isolates (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and fluorescent pseudomonads APF1, APF2, APF3, and APF4) on tomato growth response (as dry and fresh weight) as compared to untreated control under greenhouse conditions

Strain	Average dry weight per plant (g)	Increase compared to control (%)	Average fresh weight per plant (g)	Increase compared to control (%)
RP87	3.9 ab	3	30.8 bc	9
B2G	4.0 ab	5	38.7 bc	37
APF1	6.2 a	63	48.5 a	72
APF2	4.7 ab	24	42.0 abc	49
APF3	4.2 ab	11	35.2 bc	25
APF4	4.4 ab	15	32.6 bc	16
Control	3.8 b	—	28.2 c	—

Healthy plants from treatments that received antagonists and *Ralstonia solanacearum* were counted and uprooted separately and their weights compared with the untreated control that received no antagonists and *Ralstonia solanacearum*. For dry weight measurement, plants were dried in an oven at 60 °C for 3 days and weights evaluated for each treatment. Values within the same column followed by the same letter do not differ significantly ($\alpha = 0.05$) according to Tukey's mean separation test.

On the other hand, the inhibitory activity of the strains *S. setonii* RP87, *B. subtilis* B2G, and pseudomonads APF3 and APF4 was equal in LB and KB (Table 1), and amendment of KB with iron did not alter their activity significantly. From these results it may be possible to speculate that inhibitory activities of *S. setonii* RP87, *B. subtilis* B2G, and pseudomonads APF3 and APF4 were not iron-dependent. Thus antibiotic production may play an active role in the inhibition of the pathogen by these strains. Ran et al. (2005) suggested that when an antagonist is equally effective in the absence and presence of iron, antibiotics seem to be the agent of activity.

Under greenhouse conditions, plants inoculated with the antagonistic isolates pseudomonad APF1 and *B. subtilis* B2G significantly reduced disease compared to the control. Both strains reduced AUDPC by 60% and 56%, respectively. Moreover, significantly greater amounts of biomass (fresh and dry weight) compared to the control were obtained with these strains. Guo et al. (2004) reported comparable *R. solanacearum* wilt disease reduction and yield increase of tomato plants after treatment by *Bacillus* spp. and fluorescent pseudomonads. Priou et al. (2005) recorded 80% reduction of the same disease on tomato plants under greenhouse conditions using *Pseudomonas putida* (Trevisan). Antagonistic *Pseudomonas* spp. were tested for their ability to suppress bacterial wilt in tobacco and some showed promising results (Liu et al., 1999). Recently, Ran et al. (2005) reported suppression of bacterial wilt in *Eucalyptus urophylla* (Blake) by fluorescent *Pseudomonas* spp.

There are several modes of action known for rhizobacteria applied for the control of plant diseases (Blanco et al., 2004; Ran et al., 2005; Dwivedi and Johri, 2003). Pseudomonads exert a protective effect on the roots through antagonism towards phytopathogenic bacteria by producing metabolites that include: lytic enzymes (Berg, 1996); plant hormones and other plant growth promoting substances, e.g., auxins, indole-3-acetic acid, and gibberellins (Ramamoorthy and Samiyappn, 2001); siderophores (Dwivedi and Johri, 2003); and antibiotics (Dwivedi and Johri, 2003; Ran et al., 2005). *Bacillus* spp. is also known to produce a wide range of secondary metabolites such as antibiotics, non-volatile and volatile compounds (Parke and Gurian-Sherman, 2001) and lytic enzymes (Frändberg and Schnürer, 1994).

The population dynamics studies of *R. solanacearum* in the soil showed that the amount of the recoverable pathogen was significantly affected by some of the applied antagonists. At early stage of incorporation of antagonists into the soil, population of *R. solanacearum* declined in all treatments, including the control, until 3 weeks after treatment (Fig. 2). Later, however, significant differences could be observed among the treatments. In soil treated with fluorescent pseudomonad APF1 and *B. subtilis* B2G strains, density of the pathogen declined significantly and started to increase gradually. Conversely, in the presence of other strains and the control, population steadily increased. Pseudomonad APF1 strain significantly minimized the population in the soil followed by *B. subtilis* B2G compared to the control. The decline in population of the pathogen at the early stage cannot be attributed to the effect of treatments but to other factors such as death of some proportion of the population when introduced to soil from the laboratory. The population reduction observed in treatments with pseudomonad APF1 and *B. subtilis* B2G strains may be due to their antagonistic effects.

Disease development on plants (Fig. 1) was closely related to population dynamics of *R. solanacearum* in soil (Fig. 2). Disease progressed more quickly in soil treated by pseudomonads APF3 and APF4, *S. setonii* RP87, and control. However, disease increased slowly in soil treated by *B. subtilis* B2G, and pseudomonad strains APF1 and APF2 strains. Wilt incidence was significantly reduced by pseudomonad APF1 and *B. subtilis* B2G and was correlated with a decrease of the population of the pathogen in soil. Similar reports of pathogen decline in the soil due to antagonists and disease reduction have been presented (Pieterse et al., 2001; Szczech and Shoda, 2004).

The antagonist *S. setonii* RP87 that produced the highest antagonism *in vitro*, did not significantly differ from the control in both wilt suppression and biomass production in tomato. This lack of correlation between *in vitro* result and biological control *in vivo* has also been documented in other studies on other pathogens (e.g., Ran et al., 2005; Rajkumar et al., 2005). Much of inconsistency in the performance of antagonistic bacteria has been attributed to variability in the physical and chemical properties within the niches occupied by biocontrol agents, as well as the plant, that affect both colonization and expression of biocontrol mechanisms (Notz et al., 2002, Ryan et al., 2004).

Among the antagonists tested, fluorescent pseudomonad APF1 showed the most beneficial characteristics, as it consistently suppressed the *R. solanacearum* wilt and also promoted increased plant fresh and dry weight compared to untreated control (Table 3). Plant growth by rhizobacteria may be associated with secretion of auxins, gibberellins, and cytokinins (Ramamoorthy and Samiyappn, 2001) and suppression of deleterious microorganisms in the rhizosphere (Gamliel and Katan, 1993). The use of rhizosphere bacteria for increasing yield and for crop protection is an attractive approach in the modern system in developing a sustainable agriculture.

In conclusion, the fluorescent pseudomonad APF1 strain and *B. subtilis* B2G have proved to be consistently efficient in the control of *R. solanacearum* wilt disease in a *in planta* biocontrol assay under greenhouse conditions. Field studies should be undertaken to confirm the effectiveness of the antagonistic strains under natural conditions and their modes of action studied.

Acknowledgments

We are very grateful to the Catholic Academic Exchange Service (KAAD), Germany, for financial support of the first author. We also thank the Network for

the Improvement of Potato and Sweetpotato Program in East and Central Africa (PRAPACE) for financing the field research in Ethiopia. The experiments were conduct as part of a PhD study by the first author in the Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute for Biological Control, Darmstadt, with attachment to Hannover University in Germany.

References

Berg, G., 1996. Rhizobacteria of oil seed rape antagonistic to *Verticillium dahliae*. J. Plant Dis. Protec. 103, 20–30.

Bjorn, M.J., Iglewski, B.H., Ives, S.K., Sadoff, J.C., Vasil, M.L., 1978. Effect of iron on yields of exotoxin A in cultures of *Pseudomonas aeruginosa* PA-103. Infec. Immun. 19, 785–791.

Blanco, J.M., Jurado, D.R., Hervas, A., Diaz, R.M.J., 2004. Suppression of *Verticillium* wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. Biol. Control 30, 474–486.

Bloemberg, G.V., Lugtenberg, B.J.J., 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. 4, 343–350.

Boucher, C.A., Gough, C.L., Arlat, M., 1992. Molecular genetics of pathogenicity determinants of *Pseudomonas solanacearum* with special emphases on *hrp* genes. Ann. Rev. Phytopathol. 30, 443–461.

Buddenhagen, I.W., Sequeira, L., Kelman, A., 1962. Designation of races of *Pseudomonas solanacearum*. Phytopathology 52, 726.

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

Chen, W.Q., Morgan, D.P., Felts, D., Michailides, T.J., 2003. Antagonism of *Paenibacillus lentimorbus* to *Botryosphaeria dothidea* and biological control of panicle and shoot blight of pistachio. Plant Dis. 87, 359–365.

Ciampi-Panno, L., Fernandez, C., Bustamante, P., Andrade, N., Ojeda, S., Conteras, A., 1989. Biological control of bacterial wilt of potatoes caused by *Pseudomonas solanacearum*. Am. Potato J. 66, 315–332.

Dong, C., Zeng, X., Liu, Q., 1999. Biological control of tomato bacterial wilt with avirulent bacteriocinogenic strain of *Ralstonia solanacearum*. J. Sci. China Agric. Univ. 20, 1–4.

Duffy, B.K., Défago, G., 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. 65, 2438–2439.

Dwivedi, D., Johri, B.N., 2003. Antifungals from fluorescent pseudomonads: biosynthesis and regulation. Curr. Sci. 85, 1693–1703.

El Albyad, M.S., el Sayed, M.A., el Shanshoury, A.R., 1996. Effect of culture conditions on the antimicrobial activities of UV-mutants of *Streptomyces corchorusii* and *S. spiroverticillatus* against bean and banana wilt pathogens. Microbiol. Res. 151, 201–211.

Elphinstone, J.G., 1992. Virulence of isolates of *Pseudomonas solanacearum* from worldwide sources in resistant and susceptible tomato cultivars. In: Prior, P., Bart, S., Leclercq, S., Darrasse, A., Anais, G. (Eds.), Resistance to bacterial wilt in tomato as discerned by spread of *Pseudomonas (Burholderia) solanacearum* in the stem tissues. Plant. Pathol. 45, 720–726.

Englerbrecht, M.C., 1994. Modification of a semi-selective medium for the isolation and quantification *Pseudomonas solanacearum*. ACIAR Bact. Wilt News Lett. 10, 3–5.

Frändberg, E., Schnürer, C., 1994. Chitinolytic properties of *Bacillus paubli* K1. J. Appl. Bacteriol. 76, 361–367.

French, E.R., Sequeira, L., 1970. Strains of *Pseudomonas solanacearum* from Central and South America: a comparative study. Phytopathology 60, 506–512.

Gamalero, E., Lingua, G., Berta, G., Lemanceau, P., 2003. Methods for studying root colonization by introduced beneficial bacteria. Agronomie 23, 407–418.

Gamliel, A., Katan, J., 1993. Suppression of major and minor pathogens by fluorescent pseudomonads in solarized soil and non-solarized soil. Phytopathology 83, 68–75.

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.

Guo, J.H., Guo, Y.H., Zhang, L.X., Qi, H.Y., Fang, Z.D., 2001. Screening for biocontrol agents against cayenne pepper bacterial wilt. China J. Biol. Control 17, 101–106.

Guo, J.H., Qi, H.Y., Guo, Y.H., Ge, H., Gong, L.Y., Zhang, L.X., Sun, P.H., 2004. Biocontrol of tomato wilt by growth-promoting rhizobacteria. Biol. Control 29, 66–72.

Hassett, D.J., Schweizer, H.P., Ohman, D.E., 1995. *Pseudomonas aeruginosa* sodA and sodB mutants defective in manganese and iron cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. J. Bacteriol. 177, 6330–6337.

Hayward, A.C., 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bacteriol. 27, 265–277.

Hayward, A.C., 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Ann. Rev. Phytopathol. 29, 65–87.

Kang, Y., Mao, G., Lu, C., He, L., 1995. Biological control of bacterial wilt of tomato by extracellular protein defective mutant of *Pseudomonas solanacearum*. Acta Phytopathol. Sinica 22, 287–288.

Kelman, A., 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44, 693–695.

King, E.O., Ward, M.K., Raney, D.E., 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44, 301–307.

Kloepper, J.W., Leong, J., Teintze, M., Schroth, M.N., 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature 268, 885–886.

Krieg, N.R., Holt, J.G., 1984. Bergey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins, Baltimore.

Lemessa, F., Zeller, W., 2007. Isolation and characterisation of *Ralstonia solanacearum* strains from *Solanaceae* crops in Ethiopia. J. Basic Microbiol. 47, 40–49.

Lim, H., Kim, S., 1997. Role of siderophores in biocontrol of *Fusarium solani* and enhanced growth response of bean by *Pseudomonas fluorescens* GL20. J. Microbiol. Biotec. 7, 13–20.

Liu, Q.G., Li, Z., Tang, Z., Zeng, X.M., 1999. Control of tobacco bacterial wilt with antagonistic bacteria and soil amendment. China J. Biol. Control 15, 94–95.

Loper, J.E., Suslow, T.V., Schroth, M.N., 1984. Log normal distribution of bacterial populations in the rhizosphere. Phytopathology 74, 1454–1460.

McLaughlin, R.J., Sequeira, L., Weingartner, D.P., 1990. Biocontrol of bacterial wilt of potato with an avirulent strain of *Pseudomonas solanacearum*: interactions with root-knot nematodes. Am. Potato J. 67, 93–107.

Mitchell, J.K., Carter, W.E., 2000. Modeling antimicrobial activity of Clorox™ using an agar-diffusion test: a new twist on an old experiment. Bioscene 26, 9–13.

Montesinos, E., Bonaterra, A., Ophir, Y., Beer, S.V., 1996. Antagonism of selected bacterial strains to *Stemphylium vesicarium* and biological control of brown spot of pear under controlled environment conditions. Phytopathology 86, 856–863.

Notz, R., Maurhofer, M., Dubach, H., Haas, D., Defago, G., 2002. Fusaric acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHAo *in vitro* and rhizosphere of wheat. Appl. Environ. Microbiol. 68, 2229–2235.

Parke, J.L., Gurian-Sherman, D., 2001. Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. Ann. Rev. Phytopathol. 39, 225–258.

Pieterse, C.M.J., Van Pelt, J.A., Van wees, S.C.M., Ton, J., Leon-Kloosteriziel, K.M., Keurentjes, J.J.B., Verhagen, B.W.M., Knoester, M., Van der Sluis, I., Bakker, P.A.H.M., Van Loon, L.C., 2001.

Rhizobacteria-mediated induced systemic resistance: triggering, signalling and expression. Eur. J. Plant Pathol. 107, 51–61.

Priou, S., Marquez, M., Gutarra, L., 2005. Biological control of bacterial wilt of potato (*Ralstonia solanacearum*) using antagonistic *Pseudomonas putida* strains. Phytopathology 95 (Supplement) S85.

Rajkumar, M., Lee, W.H., Lee, K.J., 2005. Screening of bacterial antagonists for biological control of *Phytophthora* blight of pepper. J. Basic Microbiol. 45, 55–63.

Ramamoorthy, V., Samiyappn, R., 2001. Induction of defense related genes in *Pseudomonas fluorscens* treated chilli plants in response to infection by *Colletotrichum capsici*. J. Myc. Plant Pathol. 31, 146–155.

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. Biol. Control 32, 111–120.

Rangajaran, S., Saleena, L.M., Vasudevan, P., Nair, S., 2003. Biological suppression of rice diseases by *Pseudomonas* spp under saline soil conditions. Plant Soil 251, 73–82.

Ryan, A.D., Kinkel, L.L., Schottel, J.L., 2004. Effect of pathogen isolate, potato cultivar, and antagonist strain on potato scab severity and biological control. Bioc. Sci. Tech. 14, 301–311.

Sambrook, J., Fritsch, E.F., Maniatis, T.A., 1989. Molecular Clonning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, NY.

SAS Institute Inc., 1999. SAS Institute Inc., SAS OnlineDoc®, Version 8, Cary, NC, USA.

Silveira, E.B., Da, R., Mariano de, L.R., Michereff, S.J., 1995. Antagonism of *Bacillus* spp. Against *Pseudomonas solanacearum* and effect on tomato seedling growth. Fitopathol. Brasil 20, 605–612.

Smith, J.J., Offord, L.C., Holderness, M., Saddler, G.S., 1995. Genetic diversity of *Burkholderia solanacearum* (Synonym *Pseudomonas solanacearum*) race 3 in Kenya. Appl. Environ. Microbiol. 61, 4263–4268.

Szczech, M., Shoda, M., 2004. Biocontrol of *Rhizoctonia* damping-off of tomato by *Bacillus subtilis* combined with *Burkholderia cepacia*. J. Phytopathol. 152, 549–556.

Thomshaw, L.S., 1996. Biological control of plant pathogens. Curr. Opin. Biotech. 77, 343–347.

Toyota, K., Kimura, M., 2000. Suppresision of *Ralstonia solanacearum* in soil following colonization by other strains of *R. solanacearum*. Soil Sci. Plant Nutr. 46, 449–459.

Villacieros, M., Power, B., Sanchez-Contreras, M., Loret, J., Oruzebal, R.I., Martin, M., Franandez-Pinas, F., Bouile, I., Whelan, C., Dowling, D.N., Rivilla, R., 2003. Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meloti* in the alfalfa (*Medicago sativa*) rhizosphere. Plant Soil 251, 47–54.

Yabuuchi, E., Kosako, Y., Yano, I., Hota, H., Nishiuchi, Y., 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov., *Ralstonia solanacearum* (Smith, 1986) comb. nov. Microbiol. Immun. 39, 897–904.

Yaynu, H., 1989. Characteristics of isolates of *Pseudomonas solanacearum* in Ethiopia. Eth. J. Agric. Sci. 11, 7–13.