Evaluation of *Pseudomonas fluorescens* Isolates as Biocontrol Agents Against Bacterial Wilt Caused by *Ralstonia (Pseudomonas) solanacearum*

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Abstract

A total of 50 fluorescent pseudomonas isolates collected from different potato growing areas in Ethiopia were evaluated on King's B medium for antibiosis towards *Ralstonia solanacearum*, the pathogen of bacterial wilt of potato. Only three isolates, Pf S2, Pf Wt3 and PfW1, showed inhibitory effect on the growth of the pathogen. The three isolates were evaluated for their antagonistic effect in a greenhouse experiment. Tubers of potato clone CIP 383031.15 that was susceptible to bacterial wilt were bacteriazed in a 48 hour old culture suspension of the three isolates and Pfri, a reference strain from India, for 2 hr and planted in pots containing sterilized soil. Plants that were 2–3 leaves old were inoculated with the pathogen with a concentration of 10^6 cfu/ml. Non-dipped tubers but inoculated with the pathogen and bacteriazed and inoculated plants were used as controls. Bacteriazation of tubers with Pf S2, Pf Wt3, and PfW1 significantly reduced disease incidence compared to the pathogen-inoculated control and increased plant growth, i.e., plant height and dry weight. There was higher plant growth due to bacteriazation of tubers with PtWt3 (76.9%) followed by PtS2 (59.8%). The study findings indicated the importance of the antagonistic isolates as biological control agents and plant growth-promoting rhizobacteria.

Key words: biological control, *Pseudomonas fluorescens,* isolates, antibiosis, potato, bacterial wilt, Ethiopia

Introduction

Potato (*Solanum tuberosum L.*) is the fourth major crop of the world after rice, wheat and maize (CIP 1984). It is an excellent food source; the tuber provides high energy, quality protein and substantial amount of vitamins and minerals. Potato was introduced to Ethiopia in 1858 (Pankhurst 1964). Since then, the production of the crop has increased faster than any other food crop covering 50,000 ha (Berga 1986).

However, the yield per unit area of potato in the country is still very low compared to those of other countries like Rwanda, Egypt and Kenya. There are many factors that reduce the yield of the crop among which diseases like late blight (*Phytopthora infestans*), bacterial wilt and viruses play an important role (Yaynu 1989, Ketema 1999). Stewart (1956) reported bacterial wilt in areas around Jimma in the western part of the country.

Bacterial wilt, also known as brown rot, is caused by *Ralstonia (Pseudomonas) solanacearum* E.F. Smith, a soil-borne bacterial species. It is one of the most destructive plant diseases predominantly distributed in the tropical, subtropical and warm temperate regions of the world (Hayward 1995). The disease affects as many as 200 plant species that represent more than 50 families and are particularly members of solanaeceous such as potato, tomato, egg plant, pepper and tobacco. For example, the disease is responsible for the yield loss of

potato to the extent of 50–80% in Kenya, Burundi and Uganda (Ajanga 1993, Skoglund et al. 1993) and up to 70% in India (Sinha 1986).

In Ethiopia, the yield loss caused by the disease is not determined yet. But the disease occurs in potato growing areas of the country at higher incidences. Moreover, studies regarding the diversity of the pathogen showed that the strains belong to race 3 of biovar 2 of *R. solanacearum* (Yaynu 1989, Ketema 1999).

Different control measures of the disease are commonly employed in other countries. These include: the use of resistant variety, crop sanitation, crop rotation, selection of disease-free planting material and other cultural practices singly or in integrated pest management (IPM). However, control through the use of resistant varieties alone showed little success. Because such kind of resistance is strain-specific and liable to break down by virulent and highly polymorphic strains of *R. solanacearum* at an ambient temperature and in nematode-infested soil (Prior et al. 1994). Successful control of the pathogen through crop rotation is also not always effective. Because, as further argued by the authors, rotation practices recommended for one area may not perform well at other locations. The other reason is related to the differences in the strains involved (Prior et al. 1994).

The use of rhizosphere resident microbial antagonist, especially fluorescent pseudomonas, is noted as a promising control method. The rhizosphere is a habitat in which several biologically important processes and interactions take place primarily due to the influx of mineral nutrients from accumulation of plant root exudate from plant roots through mass flow and diffusion (Sorensen 1997, Bias 2004). Among rhizosphere resident microorganisms, fluorescent pseudomonas strains are often selected for biological control strategies because of their ability to utilize varied substrates under different conditions, short generation time and motility that assists colonization of roots. Moreover, they produce siderophore, etc, responsible for the biological suppression of several soil-borne plant pathogens (Bagnasco et al. 1998).

Sunaina et al. (1997) reported that fluorescent pseudomonas strains, when applied to potato seed tubers, were found to reduce the population of *Eriwinia cartovora* on roots and tubers by 95–100% and 28–95%, respectively. In a related study, Gamliel and Katan (1993) found that inoculation of fluorescent pseudomonas decreased the disease incidence caused by *Sclerotium rolfsii* in bean and *Fusarium* wilt in cotton and tomato. Therefore, the present study was conducted to collect isolates of *P. fluoresecens* from different potato grwoing areas of Ethiopia and evaluate their antagonistic effect against *R. solanacearum* under laboratory and greenhouse conditions.

Materials and Methods

Collection of Pseudomonas fluorescens

Soil samples were collected from potato fields in potato growing areas around Wolayita, Shashemene and Wondo Genet. *P. fluoresecens* were isolated. One gram of soil from each sample was mixed by shaking for 2 hr on a rotary shaker at 200 rpm in 100 ml of phosphate buffer solution (PBS) containing 0.88% (w/v) NaCl, 2.9 mM KH_2PO_4 and 7.1 mM K_2HPO_4. The pH was adjusted to 7.2.

PBS diluted extracts were then plated on KB medium composed of proteose peptone No.3 (Difco) 20 g, K2HPO4. $7H_2O$ 1.5 g, glycerol 15 ml and agar 15 g per liter of water which were made selective for isolation of *P. fluorescens* by adding cyclohexamide (100 µg/ml), chloroamphenicol (13 µg/ml) and ampicillin (50 µg/ml). After incubation at 28°C for 24 hr, representative types of colonies were further purified on KB agar medium and pure isolates were preserved on KB slant and stored at 4°C.

In vitro inhibition test

All isolates of *P. fluoresecens* were screened for their toxicity toward the pathogen on King's B medium (KB) agar plates in dual culture assays (Ganesan and Gnanamanickam 1987). KB plates were prepared by mixing suspension of cells scarped from 48–72 hr old culture of the pathogen with cooled and molten KB agar (42°C). The agar suspension was then dispensed into Petri dishes and allowed to solidify and spot inoculated with the test strain from a 24-hr old culture (Skathivel and Gnanamanickam, 1987). Likewise, KB agar plates spot inoculated with water were used as control. Assay plates were maintained at 28°C and observed for inhibition zones after 2 to 3 days.

The bacterial designation of isolates of *P. fluoresecens* used in the study was made based on the area of Ethiopia from where they were collected. Thus, Pfs refers to isolates of *P. fluorescens* from Shashemene area, Pfwt Wondo Genet, Pfw Wolayita, Pfb Bako, and Pfa Ambo. Likewise, Pfri refers to a reference strain of *P. fluorescens* introduced from India, while Rs262-b refers to *R. solanacearum* (pathogenic isolate) obtained from Ambo Plant Protection Research Center.

Tobacco hypersensitivity reaction

Since there is a possibility that *R. solanacearum* strains may lose their virulence and become avirulent upon storage, preliminary pathogencity and hypersensitivity tests were done to confirm their virulence and that they belonged to race 3 of *R. solanacearum*. The pathogenic isolate Rs262-b obtained from Ambo Plant Protection Research Center was injected on tobacco leaves following the method of Lazano and Sequeria (1970). Bacterial suspension of the pathogen was prepared from 48–72 hr old culture at a concentration of 10^9 cfu/ml (Klement et al. 1964). The suspension was infiltrared to the lower surface of a tobacco leaf of cv White Burley using syringe. Control plants were infiltrated with sterile distilled water. Following inoculation, plants were kept at room temperature and reactions were observed and recorded daily.

Pathogenicity test

Potato tubers of the susceptible cv CIP 383032.15 obtained from Holetta Agricultural Research Center were planted in 20 cm diameter plastic pots (one plant per pot) filled with sterilized soil. At three-leaf stage, potato plants were inoculated following the stem puncture method of Winsted and Kelman (1952) with bacterial suspension containing 10_9 cfu/ml prepared from 48–72 hr old culture at the axile of the second or third leaf from the apex. Control plants were injected with sterile distilled water. After inoculation, the plants were kept in an incubator at a temperature of 28°C for 48 hr and then transferred to greenhouse where the minimum temperature was 14.8°C and the maximum 35.2°C.

Greenhouse experiment

The greenhouse experiment was conducted at the National Soil Research Center, in Addis Ababa at an altitude of 2394 m and with minimum and maximum temperature of 19–25 °C and 25–31 °C, respectively. Based on the efficiency of in vitro antibiosis result, three isolates of *P. fluorescens* were chosen for the greenhouse experiment: Pfwt3, Pfw1 and Pfs2. The selected isolates together with Pfri, the reference strain introduced from India, were grown on KB agar medium for 24 hr and diluted to give a suspension, which was adjusted optically to concentration of 10^9 cfu/ml (OD_{600} = 1.0) (Mulya et al. 1996). Potato tuber of CIP 383032.15, which was highly susceptible to bacterial wilt, was surface-sterilized with disinfectants before treatment.

The soil was autoclaved at 121 °C for 1 hr and amended with fertilizer (N:P:K = 2:1:1)

(Mulya et al. 1996) at a rate of 10 g per pot as a solution. Sterilized soil of 3 kg was filled into plastic pots which were surface sterilized with alcohol. In each pot, two potato tubers were planted.

The treatments consisted of:

T1. inoculated with pathogen Rs262-b
T2. Pfw 1 treated (Wolayta isolate)
T3. Pfs 2 treated (Shashemene isolate)
T4. Pfwt 3 treated (Wondo Genet isolate)
T5. Pfri treated (Indian reference strain)
T6. Pfw 1 treated and Rs262-b inoculated
T7. Pfs 2 treated and Rs262-b inoculated
T8. Pfwt 3 treated and Rs262-b inoculated
T9. Pfri treated and Rs262-b inoculated
T10. Control (neither treated nor inoculated)

Potato tubers in T1 were planted in soil infested with 100 ml of 10^9cfu/ml suspension of the pathogen (Sunaina et al. 1997), while the tubers in T2 to T5 were dipped in the suspension of each antagonist isolate for 60 minutes and planted in sterilized soil. The tubers in T6 to T9 were dipped in each suspension for 60 minutes and planted in sterilized but pathogen inoculated (infested) soil. The tubers in T10 were neither dipped nor inoculated control.

The plants were watered regularly with deionized water and treatments were arranged in a completely randomized design with three replications. Observations on percentage survival and plant height were recorded. Then after, the plants were cut at the soil level and dry weights of the shoots were measured after oven dried at 60 °C for 72 hr.

The experimental data were analyzed using one-way analysis of variance. Comparison of means at 5% level was made by Tukey's test. Statistical analysis was done using SPSS software, version 12.0.

Results

Hypersensitivity reaction

Injection of bacterial suspension of Rs 262-b isolate into tobacco leaves caused yellow necrosis within 24 to 48 hr. There was no any progress of necrosis to the adjoining tissues and the plant did not wilt even after some weeks, showing hypersensitive reaction (Klement et al. 1964). In addition, the control plant injected with distilled water remained healthy.

Pathogenicity test

The result of the pathogenicity test showed that the bacterial isolate obtained from the laboratory at Ambo was pathogenic to potato. Development of wilt on the potato plant was rapid and complete wilting of the plant occurred within 8 to 13 days. Infected potato plants exhibited epinasty, stunting and browning of stems upon dissection. In addition, stem pieces cut from wilting plants produced white oozing when partially immersed in glasses of water.

In vitro antibiosis

Among the 50 local isolates of fluorescent pseudomonas evaluated for antibiosis against R. *solanacearum* on KB medium, only three were capable of inhibiting the growth of the pathogen, while all the other isolates did not produce any antibiosis (Table 1). The three isolates were: Pfwt 3 from Wondo Genet, Pfw1 from Wolayita and Pfs1 from Shashemene. Pfri, the reference strain from India, also inhibited the growth of the pathogen. The diameter of inhibition zones ranged from 1.2 to 2.4 cm. The minimum inhibition was caused by Pfri and the maximum by Pfw1 (Table 2). The isolate Pfw1 from Wolayita and Pfs2 from Shashemene produced higher zones of inhibition than the other isolates including the reference strain from India.

Table 1. In vitro antibiosis and zones of inhibition caused by fluorescent pseudomonas isolates collected from different areas of Ethiopia on R. *Solanacearum* on King's B medium

Collection area	No. of isolates	Antibiosis	Name of antagonistic isolate	Average zones of inhibition (cm)
Shashemene	10	1	PfS2	1.5
Wondo Genet	10	1	PfWt3	1.3
Wolayita	15	1	PfW1	2.4
Ambo	8	-	-	-
Bako	7	-	-	-
India	1	1	Pfri	1.2

Figure 1. Greenhouse experiment showing differences among T1 (pathogen inoculated), T6 (Pfw1 treated and Rs262-b inoculated) and T10 (non-treated control)

Table 2. Suppression of bacterial wilt caused by *R. solanacearum* in plants treated with *P. fluorescens*

Treatment	% survival*
T1. Rs262-b inoculated	40.0^{b} ± 2.3
T2. Pfw 1 + Rs 262-b	99.9^{a} ± 0.9
T3. Pfs 2 + Rs262-b	99.8^{a} ± 0.8
T4. Pfwt 3 + Rs262-b	99.8^{a} ± 1.1
T5. Pfri + Rs262-b	99.8^{a} ± 1.3
T6. Control	100^{a} ± 1.0

* Values are means and standard deviations of three replicates (two plants in each pot). Means followed by the same letter within a column are not significantly different at α = 0.05 by Tukey's test.

Greenhouse experiment

Bacterial wilt suppression by *P. Flourescens*

The findings of the experiment showed that only 40% of the plants survived in treatment T1, i.e. where potato tubers were planted in pathogen-infested soil without bacteriazation. But potato treated with the selected antagonistic isolates prior to planting of tubers in pathogen-infested soil significantly suppressed the incidence of bacterial wilt and

also increased its survival rate by 59.83% as compared to T1 (figures 1, 2 and 3). The plants in the control did not show any wilt symptom and had 100% survival.

Effect of bacteria treatment on plant height and biomass

Plant height and biomass were significantly lower in the treatment of pathogen-infested soil (T1) than all the other treatment (Table 3). Potato tuber treated with the selected isolates prior planting in pathogen free (T2–T5) and pathogen infested soil increased plant height and dry weight compared to T1. However, *P. fluorescens* treated and pathogen inoculated treatments (T6–T9) did not show increased plant height and biomass better than the control (T10).

Figure 2. Greenhouse experiment showing differences among T1 (pathogen inoculated), T8 (Pfwt3 treated and Rs 262-b inoculated) and T10 (non-treated control)

Figure 3. Greenhouse experiment showing differences among T1 (pathogen inoculated), T7 (Pfs2 treated and Rs 262-b inoculated), and T10 (non-treated control)

Table 3. Effect of bacterization with isolates of P. fluorescens on plant height and biomass

Treatment	Plant height (cm)*	Dry weight (gm)*
T1. Rs262-b ino	$20.83^{c} \pm 2.1$	$1.9^{c} \pm 1.51$
T2. Pfw1	$32.13^{a} \pm 1.1$	$11.97^{a} \pm 2.52$
T3. Pfs2	$32.87^{a} \pm 0.76$	$11.83^{a} \pm 1.19$
T4. Pfwt3	$32.83^{a} \pm 1.1$	$11.7^{a} \pm 2.41$
T5. Pfir	$32.53^{a} \pm 0.92$	$11.53^{a} \pm 0.95$
T6. Pfw1+ Rs262-b	$27.9^{b} \pm 1.8$	$6.77^{b} \pm 1.42$
T7. Pfs2+ Rs262-b	$26.77^{b} \pm 1.3$	$6.76^{b} \pm 1.2$
T8. Pfwt3+ Rs262-b	$27.17^{b} \pm 1.5$	$6.67^{b} \pm 1.12$
T9. Pfri+ Rs262-b	$26.5^{b} \pm 0.87$	$6.63^{b} \pm 1.24$
T10. Control	$27.4^{b} \pm 3.9$	$6.63^{b} \pm 1.5$

* Values are means and standard deviations of three replicates (two plants in each pot). Means followed by the same letter within a column are not significantly different by Tukey's test at $\alpha = 0.05\%$.

Discussion

Pathogenicity and hypersensitivity test

The result of the tobacco leaf infiltration test showed that the pathogen induced chlorosis of the infiltrated tobacco leaf which is the characteristics of race 3 (Marin and EI-Nashaar 1993). The result indicated that tobacco is not a host unlike race 1 of the pathogen, which causes wilting of other solanaceous crops like potato, pepper, tomato, tobacco, and eggplant. For race 3 tobacco is not a host and did not cause wilting of the plant. On the other hand, race 3 caused wilting of the potato plant within 3–6 days after inoculation, showing that the isolate Rs 262-b was a virulent and potato is the host plant. The method is, thus, one of the easy methods that distinguishes race 3 from other races of *R. solanacearum* and which confirms the virulence of long stored strains.

In vitro antibiosis

In vitro antibiosis of the isolates was tested against the target pathogen on KB medium to evaluate their antagonistic efficiency. Based on the diameter of inhibition zone, three isolates were selected for greenhouse experiment. In the current test, the smallest and larges inhibition zone of 1.2 and 2.4 mm diameter were caused by Pfri (Indian isolate) and Pfw1 (Wolayita isolate), respectively. Pfwl, with 200% efficiency, was the most efficient isolate followed by Pfs2 (125%) and Pfwt3 (111%). Savithiry and Gnanamanickam (1987) and Anuratha and Gnanamanickam (1990) also reported 2.5 to 4 cm and 1.0 to 2.8 cm diameter of inhibition zones by *P. flurescens* against *R. solanii* and *R. solanacearum*, respectively, on KB agar medium. Therefore, the three isolates in the present study were further evaluated in pot experiment under greenhouse condition.

Greenhouse experiment

The result of the greenhouse experiment showed that treatment of potato tuber with selected isolates increased the mean survival of potato plant by 59.83% compared to T1 (Table 3). The finding suggested a higher level of protection of potato from bacterial wilt and efficient isolates could be used in biological control of the disease.

Similarly, Aspiras and Cruz (1985), Anuratha and Gnanamanikam (1990), and Gamliel and Katan (1993) reported that utilization of antagonistic rhizosphere bacteria such as *Bacillus spp*, *P. fluorescens*, and *P. putida*

significantly increased the survival rate of potato, tomato, eggplant and cotton by 60–90%, 90% and 84–90%, respectively, against bacterial and fusarium wilt disease.

In the present study, bacterization of tubers with selected efficient isolates in treatments T6 to T9 significantly increased plant height by 76.89% and dry weight by 28.44% as compared to T1. The result agreed with Sivamari and Gnanamanickam (1988) who reported an increased plant height and biomass for banana seedlings by 62.17% and 61.54%, respectively, due to bacterization with the suspension of *P. fluorescens* trains prior to planting in soil infested by *F. oxysporium f.* sp *cubense*.

The increase is attributed to the fast growth rate followed by their aggressive root colonization nature that results in displacement of the pathogen and also high competitive and wide metabolic capability of the fluorescent pseudomonas isolates (Suslow 1982, Hoitink and Beohm 1999). In addition, siderophore produced by the isolates scavenge Fe (III) and induced systemic resistance and also specificity between flurescent pseudomans and potato also play a vital role in wilt suppression and growth promotion of potato (Chen et al. 2000, Whips 2001).

Potato tubers planted after bacterization in treatments T2 to T5 only with the selected antagonistic isolates showed the most significant growth enhancement (plant height and dry weight) when compared to potato plants in other treatments. The result could be explained by the possibility of production of growth stimulating substance (hormone), and nutrient solubilization nature of the isolates, suggesting that the isolates may be the member of plant growth promoting rhizobacteria (Glick 1995).

Therefore, the findings of the current sudy indicated the potential of Ethiopian *P. flourescens* isolates as biological control agents for bacterial wilt of potato bacterial wilt by exploiting the interaction between rhizosphere microorganisms. Plant protection rendered that way can be maximized by combining different methods in an integrated disease management. For instance, resistant variety and biocontrol together can give better protection than a single method. Besides, there are many untapped potentials in *P. flourescens* that should be considered for the biological control of potato bacterial wilt in the country. Some of these include: use of specific substrate that enhances selective growth and multiplication of the antagonist, use of multiple microbial inoculant rather than a single species alone, genetic manipulation of the desired promising isolate, and improving delivery of the formulation of the biocontrol agent.

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