Phenotypic Characterization of Isolates of *Ralstonia solanacearum* and Evaluation of Some Tomato Varieties Against Bacterial Wilt

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Abstract

Tomato is attacked by many bacterial diseases, among which bacterial wilt, caused by *Ralstonia solanacearum* Smith, is the most important in Ethiopia. The present study was made to characterize the pathogen phenotypically and to evaluate some tomato varieties against the disease. For phenotypic characterization, pathological, cultural, biochemical and physiological tests were done. For varietal evaluation, a greenhouse experiment was made using tomato varieties introduced from the Asian Vegetable Research and Development Center (AVRDC), Taiwan, and recently (2005) released varieties by Melkassa Agricultural Research Center (MARC), Ethiopia, for resistance against the virulent strain *R. solanacearum* PPRC-Rs. According to the results of the study, strain PPRC-Rs of *R. solanacearum* caused necrosis of tobacco leaf but not wilting; whereas, the strain caused wilting of tomato and potato. The strains of *R. solanacearum* (PPRC-Rs and 262b) were catalase and oxidase positive. Both strains of the pathogen did not hydrolyse starch and gelatin. The strains utilized glucose, lactose and cellobiose, while neither utilized dulcitol. Based on the test results, the strains were designated as race 3, biovar II of *R. solanacearum*. In addition, two AVRDC tomato varieties, BL-1004 and BL-333, showed resistant reaction to the pathogen. Among the varieties released in Ethiopia, only Fetane (Picador) showed moderate resistance (50% survived) reaction; whereas the other varieties were susceptible. The susceptible reaction of the released varieties indicated the need to evaluate the varieties against the wilt pathogen before undertaking adaptation, agronomic or any other field trial.

Key words: Bacterial wilt, *Ralstonia solanacearum*, *Lycopersicon esculentum*, potato, genotypes

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is one of the production constraints of solanaceous crops including tomato (*Lycopersicon esculentum* Mill.). Phenotypic characterization of isolates of the bacterial wilt pathogen (*Ralstonia solanacearum* Smith) was made in the mid 1980s (Yaynu 1989). There is dynamics in germplasm exchange which could be a means for introduction of new strains or races of the pathogen. Moreover, there is a need to characterize the pathogenic strain pathologically, culturally, biochemically and physiologically so as to get up-to-date phenotypic characteristics of the pathogen as the genome of some *Burcholderia cepacia* harbor many transposable elements (Lessie et al. 1996) that may cause extensive genomic and phenotypic variation of genetic material. Moreover, similar to *B. cepacia*, the genomes of *R. solanacearum* strains are composed of at least two independently replicating circular replicons: one of 3.8 Mb that apparently harbors many genes for basic cellular functions and another one of 1.9 Mb, previously called the megaplasmid, that harbors many virulence and pathogenecity genes (Boucher et al. 1986, Cook and

Sequeira 1994). Genomic flexibility and variation could relate to the diverse host range of *R. solanacearum* (Hayward 1994) and existence of biovars that, among other characteristics, vary in ability to metabolize disaccharides (cellobiose, lactose, and maltose) and hexose alcohols (mannitol, sorbitol, and dulcitol) (Buddenhagen and Kelman 1964, Hayward 1964).

Tomato (*L. lycopersicon* sub. *esculentum* Mill.) is an important solanaceous crop produced for fresh consumption as well as for processing in Ethiopia. The area under the production of the crop in the country is estimated to be 51, 698 ha and the average national yield is 4.45 t/ha (MoARD 2005). Tomato is attacked by many bacterial diseases, among which bacterial wilt is the most important in many irrigated fields in Ethiopia (Yaynu 1989, Fikre and Zeller 2007). Currently, the disease is spreading progressively to the previously unaffected areas of the country, where irrigation is used for the production of potato and tomato (Ketema 1999). Although a yield loss assessment was not carried out, the loss due to the disease is expected to be high.

Resistance is the most effective and simplest method of controlling any disease. Likewise, resistant varieties offer an effective means of controlling bacterial wilt. Opena et al. (1990) stated that both additive and non-additive gene action regulate the genetic system for wilt resistance in tomato. Developing commercially acceptable, resistant varieties has been the objective of many breeding programs (Scott et al. 1993, Young and Danesh 1994).

However, fresh market tomato varieties possessing both high resistance and high fruit quality are not available. Whereas, several highly resistant rootstocks tomato varieties have been developed in Japan (Nakaho and Takaya 1993). Growers have been grafting fresh market varieties onto resistant rootstocks to avoid bacterial wilt infection in infested areas. Grafting, however, is a time and labor consuming practice while development of highly resistant fresh market varieties is highly demanded (Monma and Sakata 1993).

There are no universal control measures which are effective across the wide host range of the pathogen (Cook et al. 1989). A control measure which is effective for one environment may not necessarily be recommended for another environment. Resistance is temperature sensitive and strain specific (French and De Lindo 1982).

Hence, there was a need to test the resistant materials introduced from Asian Vegetables Research and Development Center (AVRDC) along with the recently released improved tomato varieties against the Ethiopian virulent strain of the pathogen. Therefore, the present study was carried out to pathologically, culturally, biochemically and physiologically characterize the pathogen and to evaluate some tomato varieties against *R. solancearum* isolate.

Materials and Methods

Description of the study areas

Laboratory and greenhouse studies were conducted at the Plant Protection Research Center (PPRC), Ambo, Ethiopia, during October 2005 to November 2006. The greenhouse was adjusted for its temperature and relative humidity using air conditioner and humidity controler. Data on temperature and relative humidity were collected five times daily (at 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 were 30.5/22°C and 72.4/45.2% for the first replicate and 32.5/25°C and 70.4/40.2% for the second replicate, respectively.

Phenotypic Characterization of the Pathogen

Phenotypic characterization of the pathogen was made based on pathological, cultural, biochemical and physiological studies as described below.

Pathological characterization

Hypersensitivity and pathogenecity tests were conducted on the pathogen that was isolated from wilted potato tuber according to the methods described by Goszczynska *et al.* (2000). The hypersensitivity reaction was tested on tobacco and the pathogenecity on potato and tomato. Hypersensitivity test was carried out by infiltrating 48-hr-old-bacteria at a concentration of ~ 10^8cfu/ml into the lower surface of a mature tobacco leaf by pressing a syringe containing the suspension against the leaf, forcing the suspension into the leaf. Distilled sterile water was also used as a negative control.

Pathogenecity test was carried out by infiltrating a 48 hr-old-bacteria at a concentration of ~ 10^8cfu/ml into the stem of a 20-day old tomato transplant, L-390 (highly susceptible), and a 28-day old potato plant, Tolcha (highly susceptible), by pressing a syringe containing the suspension against the stem, forcing the suspension into the stem. Plants treated with distilled sterile water were used as a negative control.

Cultural, biochemical and physiological characterization

Cultural and morphological study

Cultural characteristics of strains of the pathogen (PPRC-Rs and 262b) were studied on different media. The media were: Tetrazolium chloride agar (TZCA) (Kelman 1954), Trypton glucose extract agar (TGA), King's B (KB) medium (King et al. 1954) and Nutrient agar (NA).

Biochemical and physiological tests

Different biochemical and physiological tests were conducted for the strains. These were: KOH solubility test (Gram reaction), Poly-β-hydroxyburate granules (PHB), catalase, oxidase, levan production, tyrosinase activity, gelatin liquefaction, starch hydrolysis and carbohydrate utilization (Fahy and Hayward 1983, Goszczynska et al. 2000, Hamouda et al. 2002).

KOH solubility test

The test was carried out by placing a drop of 3% KOH (w/v), using a Pasteur pipette on a microscope slide. Part of a single colony from agar medium was removed using sterile loop and mixed with the drop of KOH solution on the slide until an even suspension was obtained. When mucoid thread was lifted with the loop from the slide, it was designated as a gram-negative bacterium but when a completely dissolved suspension was produced, it was designated as a gram-positive bacterium (Fahy and Hayward 1983, Goszczynska et al. 2000).

Poly-β-hydroxyburate granules (PHB)

PHB staining is used to distinguish between species in the non-fluorescent pseudomonad group (Goszczynska et al. 2000). The presence of PHB, organic reserve materials in the form of polyesters of β-hydroxy butyric acid, was tested. Young colonies of strains of the pathogen were heat-fixed, PHB-stained, and counter-stained with safranine and observed under oil immersion microscopy.

Catalase test

Catalase test was performed according to the methods described by He et al. (1983). A few drops of 3% H_2O_2 were put on glass slide. Then, an 18 to 24-hr-old colony was mixed with the drops of 3% H_2O_2. Observation was made for the presence or absence of gas bubbles in the dissolved colonies, where formation of gas bubbles indicates presence of catalase activity (Goszczynska et al. 2000).

Oxidase test

Oxidase test was carried out according to Kovacs (1956) by first preparing 1 ml of 1% NNN'N-tetramethyl-p-phenylene-diamine-dihydrochloride (w/v) solution, just before

use. A few drops of the solution were put on Whatman No. 1 filter paper with a clean Pasteur pipette. Part of a young colony (18 to 24-hr-old) was then smeared with sterile toothpick on a moistened paper. Accordingly, color changes to dark purple within 30 seconds were considered as positive, while changes after more than 60 seconds were negative.

Levan production

Nutrient agar with 5% sucrose was prepared and poured to Petridishes. Then the media were streak-inoculated with a single colony of each isolate and incubated for 3 to 5 days at 28°C. Observation was made on the formation of convex, white, domed and mucoid colonies on the medium. The character could be displayed because of the activity of enzyme levan sucrase (Goszczynska et al. 2000).

Tyrosinase activity

Study of tyrosinase activity was done on a medium composed of 5 ml glycerol, 10 g Casein hydrolyzate (Oxoid), 0.5 g K_2HPO_4, 0.25 g $MgSO_4x7H_2O$, 1 g L-tyrosine and 15 g Agar; dissolved in 1000 ml of distilled water; then pH adjusted to 7.2 and autoclaved for 15 min at 121°C. The inoculated plates were incubated for 2 to 5 days at 28°C. Observation was made on whether there was red to reddish, diffusible pigment or not. The presence of the pigment was considered positive for the presence of tyrosinase activity (Goszczynska et al. 2000).

Gelatin liquefaction

Gelatin hydrolysis or liquefaction study was done by stab-inoculating tubes containing 3 g beef extract, 5 g peptone, 120 g gelatine dissolved while heating in 1000 ml of distilled water dispensed into 10 ml per tube and autoclaved for 15 min at 121°C. The inoculated test tubes were incubated for up to 15 days at 20°C. For observation on whether there was liquefaction of gelatin or not, all the inoculated test tubes and the control were kept at 5°C for 15 min before determining liquefaction (Goszczynska et al. 2000). This would make tubes with no liquefaction activity and the non-inoculated control to solidify; even then the ones with the gelatin hydrolysis would remain liquid, when tubes were tilted.

Starch hydrolysis

Starch hydrolysis study was done according to Sands (1990) by streak inoculating starch plates/nutrient agar with 0.2% soluble starch (2 g soluble starch in 1000 ml of nutrient agar dissolved and then pH adjusted to 6.8 which was finally autoclaved for 15 min at 121°C). The inoculated plates were incubated for 2 to 7 days at 28°C. Then the plates were flooded with Lugol's iodine solution (2 g potassium iodine dissolved in 25 ml, 1 g iodine added and then made up to 100 ml). As stated by Goszczynska *et al.* (2000) starch stains blue-black and a clear zone around growth indicates starch hydrolysis (amylase activity).

Oxidative/fermentative use of carbohydrates

Oxidative or fermentative use of carbohydrates was studied by stab-inoculating with bacteria taken from a young colony (18–24 hr culture) into two tubes (one covered with sterile mineral oil at the height of 1 to 2 cm and another without oil coverage, i.e, open) containing basal mineral medium plus carbohydrate. Basal medium made was of 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4x7H_2O$, 1 g Peptone dissolved in 1000 ml of distilled water, pH adjusted to 7.2 with 40% NaOH, then 0.08 g Bromothymol blue, 3 g agar and autoclaved for 15 min at 121°C, and cooled to 50°C. Finally, 5 ml of 10% filter sterile carbohydrate (w/v) was added to basal medium just before dispensing in sterile cotton plugged tubes (Goszczynska et al. 2000). The carbohydrates were: one monosaccharide (glucose), two disaccharides (lactose and cellobiose), and two sugar alcohols (mannitol and dulcitol). Sterile mineral oil was added to one of the tubes at the height of 1 to 2 cm. The inoculated tubes were incubated at 28°C for up to 3 weeks. Observation was made on whether there were color changes to yellow; the changes in only

the open tube would be labeled oxidative or in both tubes fermentative reaction.

Evaluation of some tomato varieties against R. solanacearum PPRC-Rs

Pathogenecity identification and confirmation

The identity of the pathogenic strain PPRC-Rs was ascertained using pathological, cultural, biochemical and physiological characteristics in accordance with the identification Key No. 5 by Goszczynska et al. (2000) and in comparison with the reference strain 262b of *R. solancearum* obtained from PPRC (Ketema 1999). Confirmation of the pathogenecity of strain PPRC-Rs was done by first testing the strain for hypersensitive reaction on tobacco leaf by infiltrating a 48-hr-old bacteria at a concentration of ~ 10^8cfu/ml into the lower surface of a mature tobacco leaf by pressing a syringe containing the suspension against the leaf. Pathogenecity test was carried out by infiltrating a 48-hr-old bacteria at a concentration of ~ 10^8cfu/ml into the stem of a 20-day old tomato transplant, L-390 (highly susceptible), to the disease. Then the re-isolated pathogen from the wilted tomato was used for evaluation study.

Seed source and planting procedure

Seeds of 14 tomato varieties were used for planting. Among these, 4 were introduced varieties obtained from ARFDC, Taiwan, and 10 from Melkassa Agricultural Research Center (MARC) of the Ethiopian Institute of Agricultural Research. Three of the introduced varieties were resistant while one (L390) was susceptible. In addition, the varieties from MARC were of different types, including four improved varieties released for production in 1995. Table 1 presents the experimental varieties including some brief descriptions.

The variety L390, routinely used as a susceptible control at AVRDC (Wang et al. 2000), was also used as a susceptible check in the present study and H-1350 as a standard check. The seedlings were raised in pots filled with greenhouse soil: field soil, cow dug and sand at the ratio of 2:1:1, respectively. After two weeks, the seedlings were transplanted into clay pots (20 cm diameter) with three plants per pot and were evaluated in the greenhouse randomized complete block design arrangement with four replications. The tomato evaluation experiment was done twice.

Inoculum preparation and inoculation

The *R. solanacearum* strain PPRC-Rs was grown on a tetrazolium chloride agar (TZCA) medium. Virulent (wild-type) colony, mucoid colony, was selected and increased onto Potato Dextrose Agar (PDA) and incubated for 48 hr at 28°C. Bacterial colony was gently removed from the agar surface and suspended in distilled sterile water to a concentration of ~10^8 cfu/ml by plate counting method (Humphries 1979).

Initially seedlings of the susceptible variety L390 were inoculated with the pathogenic strain. After complete wilting, the plants were removed and the pathogen was re-isolated from the wilted plants. This was done so as to refresh the virulence of the pathogen against tomato. The re-isolated bacteria were used for the rest of the evaluation study. Twenty-day-old plants were inoculated in the leaf axils using two drops (Anand et al. 1993). The inoculated plants were kept in the greenhouse and watered daily and inspected for any wilt symptom development or any other change that occurred.

Disease assessment

Disease development was assessed at 4, 7, 10, 14, 21 and 28 days after inoculation using the 0–5 disease scoring scale (Wang et al. 2000) where, 0 = no visible wilt, 1 = 1 leaf wilted, 2 = 2 or 3 leaves wilted, 3 = all except the top leaves wilted, 4 = all leaves wilted, and 5 = dead (collapsed). For wilt proportion (WP) record, the ones with disease scale of 2 to 5 were considered wilted (Wang et al. 2000). On the 100th day the spread of the pathogen in the plant was assessed by recording the degree of vascular browning by splitting stem

Table 1. Name, sources and some characteristics of the tomato varieties used in the study in 2005/2006 at Ambo-PPRC greenhouse

Variety	Source	Remark
H7996 (BL986)	AVRDC	Resistant
R-3034-3-10-NUG (BL1004)	AVRDC	Resistant
CRA 84-26-3 (BL333)	AVRDC	Resistant
L390	AVRDC	Susceptible
Bisholla (Floradado)	MARC	Released in 2005 by MARC-EIAR, tolerant to leaf diseases
Roma VF	MARC	Under production at large scale at UAAIE and Ziway state farms
Metadel (Caribo)	MARC	Released in 2005 by MARC-EIAR, tolerant to bacterial wilt
Tergeru-97	MARC	Tanzanian commercial variety
Melkasholla (Red Pear)	MARC	Processing type, released in 1997/98 by MARC-EIAR
Heinz-1350 (H-1350)	MARC	Was under production for more than 20 years and being used as standard check at MARC-EIAR
Eshet (Calypso)	MARC	Released in 2005 by MARC-EIAR,. tolerant to bacterial wilt
Fetane (Picador)	MARC	Released in 2005 by MARC-EIAR, more tolerant to leaf diseases
Marglobe	MARC	Short shelf life
Melkasalsa (Serio)	MARC	Processing type recent than Roma-VF, released in1997/98 by MARC-EIAR

PPRC, Plant Protection Research Center; AVRDC, Asian Vegetable Research and Development Center; MARC, Melkassa Agricultural Research Center; EIAR, Ethiopian Institute of Agricultural Research; UAAIE, Upper Awash Agro-Industry Enterprises.

at the root-shoot junction. Three plants per variety from each replicate were rated on the following scale: 0 = no browning; 1 = light brown color, spread restricted to 2 cm from the point of inoculation; 3 = dark brown colour, wide spread browning of vascular tissue. Presence of bacterial ooze was checked by dipping a piece of the lower cut stem from just above the root zone in a beaker with clear water. The scoring of ooze was as follows: 0 = no ooze; 1 = thin strand of ooze not continuous, flow stops after 3 min; 2 = continuous thin white, flow not restricted; 3 = heavy ooze, turning water turbid within 2 min (Anand et al. 1993).

Plants were grouped into resistance and susceptible classes using the rating: HR = highly resistant (100% survival and no browning or ooze), R = resistant (80–100% survival and no browning/browning at site of inoculation only), MR = moderately resistant (40–80% survival, slight browning and ooze), and S = susceptible (40% survival, browning and heavy ooze) (Anand et al. 1993).

Data analyses

Computer based SAS statistical software version 8 (SAS Institute 1999) was used for analysis. The value of survival percentage for tomato was transformed using arc-sine transformation (Gomez and Gomez 1984) to normalize the data for analysis as the variance was not homogenious. 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 the analysis of correlation of

traits like survival percentage, degree of vascular browning and stem ooze, PROC CORR option of ANOVA procedure was used. A significant level of $\alpha = 0.05$ was used in all analyses.

Results

Phenotypic characterization of the Pathogen

Pathological characteristics

Hypersensitive reaction and pathogenecity tests conducted on isolate PPRC-Rs indicated that it caused dry and necrotic lesion of infiltrated area within 24 hr where the reaction was clearly visible within 48 hr of inoculation, as compared to the distilled strerilized water (DSW) infiltrated control but was not pathogenic or did not cause wilting of tobacco plant. But the reference isolate of *R. solanacearum*, 262b, which is a weak pathogen, caused yellowing of infiltrated area after three weeks, which was too late as compared to the highly virulent, strain PPRC-Rs. However both strains did not cause wilting of tobacco plant.

Cultural, biochemical and physiological characteristics of R. solanacearum strains (PPRC-Rs and 262b)

Cultural and morphological characteristics

Both strains of the pathogen showed mucoid, drop-shaped white colonies with pink centers on TZCA. When observed under microscope, the cells were motile and rod-shaped. On TGA mucoid white colonies were observed. The strains did not produce fluorescien color on KB. On NA the strains showed gray mucoid colonies.

Biochemical tests

R. solanacearum strains did not dissolve in

Table 2. Biochemical and physiological characteristics of *R. solanacearum* strains

Test	*R. solanacearum* strain [1]			
	PPRC-Rs		262b	
Biochemical:				
KOH solubility (Gram reaction)	-		-	
Poly-β-hydroxyburate granules (PHB)	+		+	
Catalase	+		+	
Oxidase	+		+	
Levan production	-		-	
Tyrosinase activity	-		-	
Physiological:				
Decomposition of macromolecules:				
Gelatin liquefaction	-		-	
Starch hydrolysis	-		-	
Utilization of:				
Glucose	O+	F-	O+	F-
Lactose	O+	F-	O+	F-
Cellobiose	O+	F-	O+	F-
Mannitol	-	-	-	-
Dulcitol	-	-	-	-
Biovar designation:	Bv-2		Bv-2	

1: -, no/negative reaction; +, positive reaction; F+, fermentative reaction/anaerobic growth (where both the 'open' and the oil covered test tubes turned yellow), and O+, Oxidative reaction/aerobic growth (where only the open test tubes turned yellow and the oil covered ones remained dark-green) (Goszczynska et al., 2000).

3% KOH solution, they rather showed a thin strand of slime when the mixed bacterial culture in the solution was lifted with the inoculating loop and hence were gram negative. As for the presence or absence of Poly-β-hydroxyburate granules (PHB), blue black granules in pink cells were observed which indicated that the strains accumulated the organic reserve materials in the form of PHB. The two strains of *R. solanacearum* formed gas bubbles when a 24-hr-old colony mixed in a few drops of 3% H_2O_2; hence, the strains were catalase positive. The strains of *R. solanacearum* were oxidase positive, as they showed purple color within 8 seconds after mixing of culture to the oxidase reagent. The strains failed to form levan through the action of levan sucrase (Table 2).

Physiological tests

When tubes were tilted, liquefaction was not observed in all pathogen-inoculated ones,

Table 3. Reaction of tomato varieties to bacterial wilt pathogen *R. solanacearum* PPRC

Variety	Survival (%)	Vascular browning[a]	Stem ooze[b]	Reaction to bacteria wilt[c]	Growth character
H7996 (BL-986)	54.73b (66.66)	1.00de	0.75c	MR	Indeterminate
R-3034-310-NUG (BL-1004)	88.38a (100)	0.25e	0.00d	R	Determinate
CRA 84-26-3 (BL-333)	88.38a (100)	0.50e	0.00d	R	Semi determinate
L390 (SC)[c]	1.65c (0.00)	3.00a	3.00a	HS	Indeterminate
Bisholla (Florado)	1.65c (0.00)	3.00a	2.50a	S	Determinate
Roma VF	1.65c (0.00)	3.00a	2.50a	S	Determinate
Metadel (Caribo)	1.65c (0.00)	3.00a	2.75a	S	Indeterminate
Tengeru-97	10.053c (8.33)	1.50cd	3.00a	S	Determinate
Melkasholla (Red Pear)	1.65c (0.00)	2.5ab	2.50a	S	Determinate
Heinz-1350 (Std.C)[d]	1.65c (0.00)	3.00a	3.00a	HS	Determinate
Eshet (Calypso)	10.053c (8.33)	3.00a	2.75a	S	Indeterminate
Fetane (Picador)	44.99b (49.99)	2.00bc	1.50b	MR	Determinate
Marglobe	1.65c (0.00)	3.00a	3.00a	S	Indeterminate
Melkasalsa (Serio)	10.053c (8.33)	2.50ab	2.75a	S	Determinate
CV (%)	35.6	27.4	20.5		

[1]Means within columns followed by the same letter(s) are not significantly different at (α = 0.05) using Duncan Multiple Range Test. Values in parenthesis are the original/untransformed data.[a] Length of vascular browning scale (0-3). [b] Degree of stem ooze scale (0-3). [c] Susceptible check. [d] Standard check. HR = highly resistant (100% survival and no vascular browning or ooze), R = resistant (80-100% survival and no browning / browning at site of inoculation only), MR = moderately resistant (40-80% survival, slight browning and ooze), and S = susceptible (40% survival, browning and heavy ooze) (Anand *et al.*, 1993). HS = highly susceptible (<40% survival, with dark vascular brown colour and heavy ooze).

Table 4. Estimates of correlation coefficients (r) for plant survival

Character	Survival (%)	Vascular browning	Stem ooze
Survival (%)	**1**	**-0.96622****	**-0.95081****
Vascular browning		**1**	**0.98082****
Stem ooze			**1**

** Significant at 1% level

which remained solidified. The strains of the pathogen did not show clear zones around their growth on NA with 5% starch. As to the oxidative/fermentative use of carbohydrates, the strains showed oxidative utilization of glucose, lactose and cellobiose. The strains oxidatively metabolized glucose confirming that the strains are aerobic. As to sugar alcohol, mannitol both strains did not utilize mannitol in the aerobic and anaerobic tubes. However, neither strains of the pathogen utilized the sugar alcohol, dulcitol (Table 2).

Evaluation of varieties

Identification and confirmation of the pathogenecity of PPRC-Rs

Typical colony (white colonies with pink centers) of *R. solanacearum* on its characterizing medium (TZCA) were observed for both strains PPRC-Rs and 262b of the pathogen on the medium. Strain PPRC-Rs caused necrotic lesion on tobacco within 24 hr, where the symptom was clearly visible in 48 hr, and caused wilting of potato and tomato within 4-8 days after inoculation. The strain accumulated PHB, did not form levan from sucrose, and did not hydrolys starch and gelatine; but, it was oxidase and catalase positive (Table 2). Hence, strain PPRC-Rs was identified as *R. solanacearum*. The re-isolated pathogen from wilted tomato was used for tomato evaluation study.

Greenhouse study

Survival percentage of the evaluated tomato varieties ranged from 0 to 100% (Table 3). None of the tested varieties showed complete resistance with 100% survival, with no browning and stem ooze. Tomato variety BL-1004 and BL-333 showed resistance reaction (100% survival) with slight vascular browning and no ooze. The moderately resistant variety BL-986 showed 66.66% survival, 1 score for browning and 0.75 for ooze. Among the four tomato varieties released in 2005 from Ethiopia, only Fetane (Picador) showed moderate resistance reaction to the pathogen with 50% survival, 2 score for browning and 1.5 for ooze (Table 3) indices.

Correlation of survival of varieties with vascular browning and stem ooze

The survival of plants after 100 days was negatively highly significantly ($P < 0.0001$) correlated with indices of stem vascular browning and ooze with correlation coefficient (r) of -0.96622 and -0.95081, respectively. However, indicies of stem vascular browning and ooze were highly significantly ($P < 0.0001$) correlated to each other with correlation coefficient (r) of 0.98082 (Table 4). The results indicated the direct relationship between the three characters. Bacterial infection first causes vascular browning and then it multiplies which leads to bacterial ooze and then results in partial or whole wilting of the plant and finally collapse of the whole plant (McGravey et al. 1999).

Discussion

R. solanacearum PPRC-Rs was not pathogenic as it did not cause wilting of tobacco plant; it caused only necrosis. The reference isolate PPRC-262b also did not cause necrosis or wilting but yellowing of the infiltrated area on tobacco, indicating that tobacco is not the host plant for both isolates which is the characteristics of race 3 of the pathogen. Hence, both strains belonged to pathogenic group III and race 3 of the pathogen in accordance with Marin and El-Nashaar (1993) and Fikre and Zeller (2007).

The cultural characteristics of both strains of the pathogen displayed on TZCA were the same as the other isolates of Ethiopia studied by Fikre and Zeller (2007) and were in line with the description of the cultural characteristics of the pathogen on TZCA, TGA and KB by Goszczynska et al. (2000).

Both strains of the pathogen did not dissolve in 3% KOH which is an indication of the gram negative character of the strains. The

reaction shown by the strains of the pathogen agreed with all previous works by He et al. (1983), Yaynu (1989) and recently by Fikre and Zeller (2007). In both strains, blue black granules in pink cells were observed. The observation indicated that the strains accumulate the organic reserve materials in the form of PHB. The result was in line with the descriptions of PHB positive by Goszczynska et al. (2000). The two strains of *R. solanacearum* formed gas bubbles. Therefore, the strains hade the capacity to break down 3% H_2O_2 into H_2O and O_2. The catalase positive character of *R. solanacearum* was also reported by Fikre and Zeller (2007). The result was also in consistency with the description given by Goszczynska et al. (2000) for catalase positive bacteria. The oxidase-positive character of the pathogen was also reported by authors like He et al. (1983), Yaynu (1989) and Fikre and Zeller (2007).

The strains failed to form levan from sucrose. The result indicated that the strains did not have the enzyme levan sucrase which would help the microbes to utilize sucrose and form levan. Hence, the strains failed to fulfill the description of levan positive bacteria as stated by Goszczynska et al. (2000).

The strains did not hydrolyse the macromolecule gelatine. This is one of the peculiar characteristics of *R. solanacearum*. The result was in agreement with Fikre and Zeller (2007). Amylase activity was not observed in the strains. The absence of amylase activity in *R. solanacearum* or the inability of the pathogen to hydrolyze starch was also reported by several authors like He et al. (1983), Yaynu (1989) and Fikre and Zeller (2007). Hence, the strains are not capable of hydrolyzing both gelatine and starch.

As to the oxidative/fermentative use of carbohydrates, the strains oxidatively metabolized glucose, confirming the strains as aerobic. A similar result was also reported by Yaynu (1989) and more recently by Fikre and Zeller (2007) who tested for bv I, bv II, and bv III of the pathogen. The sugar alcohols, mannitol and dulcitol were not utilized by both strains in neither of the tubes. According to the results, therefore, the strains belong to the biovar II (bv II) of the pathogen, the biovar which is characterized by its inability to utilize sugar alcohols (Goszczynska et al. 2000). This characteristic of the pathogen was also reported by Yaynu (1989) and by Fikre and Zeller (2007) for bv I and bv II of the pathogen. Whereas, bv III, bv IV and bv V can utilize mannitol. However, according to Goszczynska et al. (2000) and Fikre and Zeller (2007), bv III and bv IV of the pathogen can utilize dulcitol. The different biovars of *R. solanacearum* showed similar reaction to the three disaccharides (Goszczynska et al. 2000). As stated for maltose all the biovars of *R. solanacearum* have similar reaction to all sugar-alcohols except bv V (Goszczynska et al. 2000). Hence, the absence of such chemicals may not have significant effect on the physiological characterization of the pathogen.

In conclusion of the characterization study, the pathogenic strain *R. solanacearum* PPRC-Rs belonged to pathogenic group III (G-III), race-3 and bv II. Hence, after about a quarter of a century, there was no shift of races as studied previously (Yaynu 1989). Based on the results of the present study using two strains, it seems that the pathogen did not undergo phenotypic evoloution since then, or there was introduction of other races.

According to the results of the varietal evaluation for resistance to bacterial wilt, tomato variety BL-1004 and BL-333 were resistant with no wilt and no ooze. The result showed the ability of the two varieties to reduce the multiplication of the pathogen inside the plants. The varieties reported by AVRDC as resistant maintained their resistance reaction for the Ethiopian virulent strain of the pathogen. Such phenomenon is rare; because resistance is usually dependent on environment, geographic position, and

strain of the pathogen. Therefore, the results indicated the good potential of the varietites to be used in breeding programs or as components of an integreted disease management (IDM) package for bacterial wilt in Ethiopia.

The variety BL-986 formerly reported to be resistant by AVRDC, was moderately resistant in the present study. The result showed that the variety failed to fully maintain its resistance against the strain used. Resistance could be temperature sensitive and strain specific (French and De Lindo 1982). However, variety BL-986 had better resistance than the moderately resistant variety IHR 821 which showed 57.1% survival and a score of 2 for vascular browning and 1.1 for ooze, as reported by Anand et al. (1993).

Among the four tomato varieties released in 2005, only Fetane (Picador) showed moderate resistance reaction to the pathogen. In addition, the variety Fetane showed less survival percentage than BL-986 and the moderately resistant variety IHR 821 as reported by Anand et al. (1993). The ooze index for Fetane was more than 1.0, indicating that the bacteria was able to multiply in the variety without causing total collapse of the whole plant population. A similar finding was also reported by Nirmaladevi and Tikoo (1993) where bacterial wilt resistant variety had even shown ooze index of more than 1.0, indicating the ability of the bacteria to multiply in the varieties without killing the plants. However, the rest of the tested varieties including the recently released resistant varieties were all susceptible to bacterial wilt pathogen. In particular, variety Marglobe showed high susceptibility reaction similar to the susceptible control variety L390. Therefore, before release, tomato germplasms should be subjected to a screening trial against the major diseases of the crop.

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References

Anand N., Sadashiva AT., Tiko SK., Ramkishun K., Red Monma, Madhavi S., Sakata Y. 1993. Resistance to Bacterial Wilt in Tomato: Gene Dosage Effects. In: *Bacterial Wilt.* eds. Hartman GL, Hayward AC., pp. 142-148. ACIAR Proceedings No. 45.

Boucher CA, Martinel A., Barberis P., Alloing G., Zischek C. 1986. Virulence genes are carried by a megaplasmid of the plant pathogen *Pseudomonas solanacearum*. *Mol. Gen. Genet.* **205:** 270-75.

Buddenhagen IW, Kelman A. 1964. Biological and physiological aspects of bacterial wilt casused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* **2:** 203-30.

Cook D., Barlow E., Sequeira L. 1989. Genetic diversity of *Pesudomonas solanacearum*: Detection of restiriction fragment length polymorphisims with DNA probes that specify virulence and the hypersensitive response. *Mol. Plant-Microbe Interact.* **2:** 113-21.

Cook D., Sequeira L. 1994. Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. In *Bacterial Wilt: The Disease and Its Causative Agent Pseudomonas solanacearum*. eds. A.C. Hayward, G.L. Hartman, pp. 77-94. CAB Int., Oxford.

Fahy P.C., Hayward A.C. 1983. Media and methods for isolation and diagnostic tests. *In*: Methods in Phytobacteriology. Klement Z., Rudolph K., Sands D.C. (eds.). Akademiai Kiado, Budapest, Hungary.

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

French ER., De Lindo L. 1982. Resistance to *Pseudomonas solancearum* in potato: strain specificity and temperature sensitivity. *Phytopathology* **72:** 1408-1412.

Gomez KA., Gomez AA. 1984. Statistical procedures for agricultural research. 2nd Edition. John Wiley and Sons, New York. 680p.

Goszczynska T., Sefontein JJ., Serfontein S. 2000. Introduction to Practical Phytobacteriology. ARC-Plant Protection Research Institute. Pretoria, South Africa. 83p.

Hamouda T., Shih AY., Baker JR. 2002. A rapid staining technique for the detection of the initiation of germination of bacterial spores. *Letters in Applied Microbiology* **34:** 86-90.

Hayward AC. 1964. Characteristics of *Pseudomonas solanacearum*. *J. Appl. Bacteriol.* **27:** 265-77.

Hayward AC. 1994. The hosts of *Pseudomonas solanacearum*. In *Bacterial Wilt*: *The Disease and its Causative Agent Pseudomonas solanacearum*. eds. Hayward, A.C., Hartman, G.L., pp. 9-24. CAB Int.,Oxford.

He LY., Sequeira L., Kelman A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. *Plant Disease* **67:** 1357-1361.

Humphries J. 1979. Bacteriology. Pitman Press, UK, London. 88pp.

Ketema Abebe. 1999. Bacterial Wilt *Ralstonia* (*Pseudomonas*) *solanacearum* of Potato in South and Central Ethiopia: Distribution, latency and pathogen characterization. M.Sc Thesis, Addis Ababa University, Addis Ababa, Ethiopia. 83p.

King EO., Ward MK., Raney DE. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine*. **44:** 301-307.

Kovacs L. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature* **178:** 703.

Lessie TG., Hendrickson W., Manning B., Devereux R. 1996. Genomic complexity and plasticity of *Burkholderia cepacia*. *FEMS Microbiol. Lett.* **144:** 117-28.

Marin JE., El-Nashaar NM. 1993. Pathogenecity of new phenotypes of *Pseudomonas solanacearum* from Peru. In: *Bacterial Wilt*. eds. Hartman GL., Hayward AC., pp. 78-84. ACIAR Proceedings No. 45.

McGarvey J., Denny TP., Schell MA. 1999. Spatial-temporal and quantitative analysis of growth and EPS production by *Ralstonia solanacearum* in resistant and susceptible tomato varieties. *Phytopathology* **89:** 1233-39.

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

Monma S., Sakata Y. 1993. Inheritance of Resistance to Bacterial Wilt in Tomato. In: *Bacterial Wilt*. eds. Hartman GL., Hayward AC., pp. 149-153. ACIAR Proceedings No. 45.

Nakaho K., Takaya S. 1993. Resistance of Tomato Rootstock Cultivars to *Pseudomonas solanacearum* Evaluated by Infection Rate Under Different Testing Conditions. In: *Bacterial Wilt*. eds. Hartman GL. Hayward AC., pp. 138-141. ACIAR Proceedings No. 45.

Nirmaladevi S., Tiko S.K. 1993. Studies on Genetic Resistance to Bacterial Wilt and Root-Knot Nematode in Tomato. In: *Bacterial Wilt*. eds. Hartman GL., Hayward AC., pp. 163-169. ACIAR Proceedings No. 45.

Opena RT., Hartman GL., Chen JT., Yang CH. 1990. Breeding for bacterial wilt resistance in tropical tomato. In: 3rd International Conference on Plant Protection in the Tropics. 1990 *Malaysian Plant Protection Society* **5:** 44-50.

Sands DC. 1990. Physiological criteria-determinative tests. In: *Methods in Phytobacteriology*. eds. Klement Z., Rudolph K., Sands DC., pp. 134-143. Akademiai Kiado, Budapest, Hungary.

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

Scott JW., Somodi GC., Jones JB. 1993. Testing Tomato Genotypes and Breeding for Resistance to Bacterial Wilt in Florida. In: *Bacterial Wilt*. eds. Hartman GL., Hayward AC., pp. 126-131. ACIAR Proceedings No. 45.

Wang Jaw-Fen, Olivier Jocelyne, Thoque Philippe, Mangin Brigitte, Sauviac Laurent, Grimsely Nigel H. 2000. Resistance of Tomato

Line Hawaii 7996 to *Ralstonia solanacearum* Pss4 in Taiwan Is Controlled Mainly by a Major Strain-Specific Locus. *Mol. Plant-Microbe Interact.* **12 (1):** 6-13.

Yaynu Hiskias. 1989. Characteristics of isolates of *Pseudomonas solanacearum* in Ethiopia. *Ethiopian Journal of Agricultural Sciences* **11:** 7-13.

Young ND., Danesh D. 1994. Understanding Bacterial Wilt Resistance in Tomato Through the Use of DNA Genetic Markers. In: *Bacterial wilt: the Disease and its Causative* Agent *Pseudomonas solanacearum.* eds. Hayward AC., Hartman GL., pp. 145-156. CAB Int., Oxford.