Biochemical, Pathological and Genetic Characterization of Strains of *Ralstonia solanacearum* (Smith) from Ethiopia and Biocontrol of *R. solanacearum* with Bacterial Antagonists

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Dedicated to my parents

Abstract

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Fikre Lemessa Ocho

Ralstonia solanacearum (Smith) is a very destructive bacterial plant pathogen that causes wilt disease in potato, tomato and other solanaceae crops in Ethiopia. The strains of this heterogeneous bacterial species differ in biochemical, pathogenic and genetic characteristics and no effective control measure has been developed against it yet. Thus the general objectives of the present study were to: (i) determine the biochemical and pathogenic characteristics and know the available biovars and races of the pathogen in Ethiopia, (ii) assess the genetic diversity of Ethiopian strains, and (iii) screen bacterial antagonists for selection of effective biocontrol agents. For characterization and genetic diversity study, 62 strains collected from Ethiopia and five reference strains were used, while for screening antagonists, 118 rhizospheric bacteria collected from Ethiopia and 20 obtained from Rostock University, Germany, were used. Six antagonistic bacteria were selected for greenhouse test on *in vitro* basis.

Based on biochemical and pathogenic characteristics, Ethiopian *R. solanacearum* strains were grouped into biovar I/race 1 and biovar II/race 3. As biovar I/race 1 *R. solanacearum* strains had not been reported from Ethiopia previously, they were detected for the first time during this study. When the effect of temperature regimes on growth of three biovars (I, II, and III) was examined, variability among biovars was observed. Generally, biovar I and III strains had a good growth at high temperature (37°C), while biovar II at lower temperature (22°C). When the effect of age of host inoculation (1-2, 3-4, and 5-6 true leaf stage) on development of wilt disease by two races (1 and 2) was tested, disease development varied depending on the type of host and race of the pathogen. In general, age of host inoculation did not affect wilt development in potato, tomato, and eggplant for both race 1 and 3, while in pepper and tobacco plant inoculation at 5-6 true leaf stage reduced wilt development by race 1, indicating the existence of age related resistance against the pathogen.

Studies on genetic diversity by repetitive sequence-based polymerase chain reaction (rep-PCR) defined two major groups among Ethiopian strains at 55% similarity level, where each group correlated with a biovar. Furthermore, at 90% similarity level, it showed diversity in biovar II strains and homogeneity in biovar I strains of Ethiopia. In general, the result showed genetic variation among Ethiopian strains. This indicates the importance of designing control measure and breeding programs based on this variability.

In the biocontrol studies six rhizospheric bacteria were selected after *in vitro* test and variability among strains was observed in wilt suppression in a greenhouse experiment. The most effective strains (fluorescent pseudomonad APF1 and *Bacillus subtilis* B2G) consistently reduced wilt disease and increased plant weight significantly. The pseudomonad APF1 strain showed the greatest plant growth promotion effect, increasing plant dry weight up to 63% compared to untreated control. The mode of action of pseudomonad APF1 strain was partly due to siderophore production.

In summary, the results of the study could clearly show that there are two major distinct groups of *R. solanacearum* strains in Ethiopia which are different in biochemical, pathogenic and genetic characteristics. This suggests that control methods should be developed taking the diversity into consideration. Moreover, pseudomonad APF1 and *B. subtilis* B2G strains could be selected as potential antagonists that should be further tested under field condition for their efficacy.

Keywords: *Ralstonia solanacearum*, biochemical characteristics, pathogenic characteristics, genetic diversity, biological control

Zusammenfassung

Biochemische, Pathologische und Genetische Charakterisierung von Stämmen von *Ralstonia solanacearum* (Smith), den Erreger der bakteriellen Welke, in Äthiopien und ihre biologische Kontrolle mit bakteriellen Antagonisten

Fikre Lemessa Ocho

Ralstonia solanacearum (Smith), der Erreger der bakteriellen Welke, ist ein sehr gefährlicher Krankheitserreger, der in Äthiopien an Kartoffel, Tomate und anderen Pflanzen aus der Familie der Solanaceae vorkommt. Die Bakterien-Species unterscheidet sich in biochemischen, pathogenen und genetischen Eigenschaften; und bisher wurde keine wirksame Bekämpfungsmaßnahme gegen diese entwickelt. Das Ziel der vorgelegten Arbeit ist: (i) in Äthiopien befindliche Biovare und Rassen des Krankheitserregers zu isolieren und deren physiologische und pathogene Eigenschaften zu bestimmen, (ii) die genetische Diversität des Bakteriums aufzuklären und (iii) wirksame bakterielle Antagonisten zur biologischen Kontrolle der Krankheit zu isolieren. Zur Charakterisierung des Erregers wurden 62 Stämme aus Äthiopien isoliert und mit fünf bekannten Stämmen als Kontrolle verglichen. Zur Selektion der Antagonisten wurden 118 Bakterien der Rhizosphäre aus Äthiopien isoliert und 20 weitere, die von der Universität Rostock, Deutschland zur Verfügung gestellt wurden, verwendet. Auf der Grundlage der *in vitro* -Experimente wurden 6 Antagonisten ausgewählt und für die *in planta* Versuche im Gewächshaus verwendet.

Die *R. solanacearum* Stämme aus Äthiopien wurden auf der Grundlage der biochemische und pathologischen Eigenschaften in Biovar I/Rasse 1 und Biovar II/Rasse 3 eingruppiert. Von einem Vorkommen von Biovar I/Rasse 1-Stämmen in Äthiopien war bisher nichts bekannt. Sie wurden erst an Hand dieser Studie entdeckt. Untersuchungen zur Temperaturabhängigkeit des Wachstums von drei Biovaren (I, II, und III) zeigten, dass es unter diesen eine Variabilität gibt. Generell zeigten Biovar I und III-Stämme ein gutes Wachstum bei höherer Temperatur (37°C), Biovar II Stämme bei niedrigerer Temperatur (22°C). Es wurde ein Zusammenhang zwischen dem Alter der Wirtspflanze (1-2, 3-4, und 5-6 Blattstadium) und der Krankheitsentwicklung nachwiesen. Die Entwicklung der Welke variierte in Abhängigkeit vom Wirtstyp und von der Rasse des Pathogens. Das Alter der Pflanze von Kartoffel, Tomate, und Aubergine hatte bei der Inokulation sowohl von Rasse 1 als auch von Rasse 3

keinen Einfluss auf die Welkeentwicklung, demgegenüber jedoch bei Paprika und Tabakpflanzen. Diese Pflanzen zeigten eine geringere Welke durch die Rasse 1 während des 5-6 Blattstadiuns. Dieses Ergebnis zeigt, dass es bei den Paprika- und Tabakpflanzen eine altersabhängige Resistenz gegen den Erreger bei der Rasse I gibt.

Untersuchungen in Hinsicht auf die genetische Variabilität der Stamme wurde mit Hilfe der repetitiven Polymerase-Kettenreaktion (rep-PCR), durchgeführt. Es wurden zwei Hauptgruppen der äthiopischen *R. solanacearum*-Stämme festgstellt, die eine Ähnlichkeit von 55 % aufwiesen, wobei jede dieser Gruppen mit einem Biovar korrelierte. Zu 90% zeigten die Stämme von Biovar 1 ein homogenes Verhalten, während bei denen von Biovar II eine Diversität festzustellen war. Im Allgemeinen zeigt dieses Ergebnis, dass eine genetische Variablität der äthiopischen *R. solanacearum*-Stämme vorliegt. Das weist auch darauf hin, dass diese Variabilität bei der Planung und Entwicklung von Pflanzenschutzmaßnahmen und Züchtungsprogrammen besondere Berücksichtigung finden sollte.

In den Untersuchungen zur biologischen Bekämpfung der bakteriellen Welke wurde nach dem *in vitro* Test 6 Rhizosphärebakterien selektiert und nachfolgend im Gewächshaus-Versuch ein unterschiedliches Verhalten der Stämme in der Reduktion der Welke festgestellt. Die wirksamsten Bakterienstämme (fluorescenter *Pseudomonas* Stamm APF1 und *Bacillus subtilis* B2G) reduzierten die Welke und erhöhten deutlich das Pflanzengewicht. *Pseudomonas* APF1 zeigte die größte fördernde Wirkung mit einer Erhöhung des Trockengewichtes von 63% im Vergleich zur unbehandelten Kontrolle. Diese Wirkung von *Pseudomonas* APF1 beruhte teilweise auf der Produktion von Siderophoren.

Zusammengefasst zeigten die Ergebnisse dieser Untersuchungen, dass es zwei verschiedene Hauptgruppen von *R. solanacearum*-Stämmen in Äthiopien gibt, die sich in physiologischen, pathogenen und genetischen Eigenschaften unterscheiden. Das bedeutet, dass Bekämpfungsmaßnahmen unter Berücksichtigung der Variabilität entwickelt werden sollten. Außerdem wurden *Pseudomonas* APF1 und *B. subtilis* B2G-Stämme als potentielle Antagonisten selektiert, die auf ihre Wirkung unter Feldbedingungen noch weiter geprüft werden sollten, um sie für eine biologische Bekämpfung später einsetzen zu können.

Schlüsselwörter: *Ralstonia solanacearum*, biochemische Eigenschaften, pathogene Eigenschaften, genetische Variabilität, biologische Bekämpfung,

Table of Contents

Abbreviations

ANOVA	Analysis of variance
bp	Base pairs
°C	Degree Celsius
cfu	Colony forming unit
cv	Cultivar
dDNTP	Dinuclo tri-phosphate
df	Degree of freedom
DNA	Deoxyribonucleic acid
F	Statistical *F*- value
Fig.	Figure
g	Gram
GLM	General linear model
h	Hour(s)
µl	Microlitre
µM	Micromolar
mg	Milligram
min	Minute(s)
ml	Millilitre
mm	Millimetre
ns	Non-significant
P	*P*-value (statistical significance level)
pmol	Picromole
r	Correlation coefficient
rpm	Revolution per minute
s	Second(s)
SAS	Statistical analysis system
SE	Standard error
spp.	Species
w/v	Weight by volume
%	Per cent

1 General Introduction

Ralstonia solanacearum (Smith) (Yabucchi *et al.,* 1995) (synonyms: *Pseudomonas solanacearum* [Smith] Smith and *Burkholderia solanacearum* [Smith] [Yabuuchi *et al.,* 1992]) is the causal agent of bacterial wilt disease in many plants (Hayward, 1995). The genus *Ralstonia* has been classified in the beta subclass of the *Proteobacteria* (Kersters *et al.,* 1996) and falls within rRNA homology group II of the taxon *Pseudomonas* (Palleroni *et al.,* 1973). In a taxonomic study of certain non-fluorescent species of the genus *Pseudomonas* (Yabuuchi *et al.,* 1992), the genus *Burkholderia* was proposed to encompass the variation found in this group and the name *Burkholderia solanacearum* was proposed. Subsequent study of this genus revealed that *Ralstonia solanacearum* was sufficiently distinct from other members of the genus to warrant assignment to the newly proposed genus *Ralstonia* (Yabuuchi *et al.,* 1995).

The species *R. solanacearum* is a severe and devastating disease affecting many crops in tropical, subtropical and some relatively warm temperate regions of the world, where it causes large crop losses (Hayward, 1991). Recently, the geographical spectrum has been extended to more temperate countries in Europe and North America as the result of the dissemination of strains adapted to cooler environmental conditions (Genin and Boucher, 2004). Symptoms of *R. solanacearum* include leaf yellowing, wilting, and necrosis, as well as vascular browning (Swanson *et al.,* 2005) (Fig. 1.1). Typically, stem and tuber cross-sections ooze a whitish bacterial exudate (Genin and Boucher, 2002). The bacterium survives in infected plants, volunteer crops, susceptible weed hosts and infested soil. Its dissemination is mainly through use of infected plants, latently infected planting material, and contaminated irrigation water (Hayward, 1991; Hayward, 1994).

The bacterium is a complex taxonomic unit in which strains display an important diversity in host range, pathogenicity, biochemical/physiological properties, geographical distribution, and epidemiological relationships (Poussier *et al.,* 1999; Horita and Tsuchiya, 2001). In order to describe this intraspecific variability, several systems of classification have been proposed. Thus the species was subdivided into five races according to its host range (Buddenhagen *et al.,* 1962; Pegg and Moffett, 1971; He *et al.,* 1983) and into six biovars based on the utilization of three disaccharides and three hexose alcohols (Hayward, 1964; He *et al.,* 1983; Hayward *et al.,* 1990). The race and biovar classification does not correspond, except that race 3 is generally equivalent to biovar II (Hayward, 1991). In addition to race and biovar classification systems, another classification scheme based on restriction fragment length polymorphism (RFLP) and other genetic finger printing studies (Hayward, 2000) have provided a new classification scheme and divided the species into two majour divisions reflecting the phylogenetic relationships. Division I contained all members of biovars III, IV, and V originating in Asia and Australia, whereas division II included all members of biovars I and II originating in South America. Recently, a third group of strains of African origin has also been identified (Poussier *et al.,* 2000).

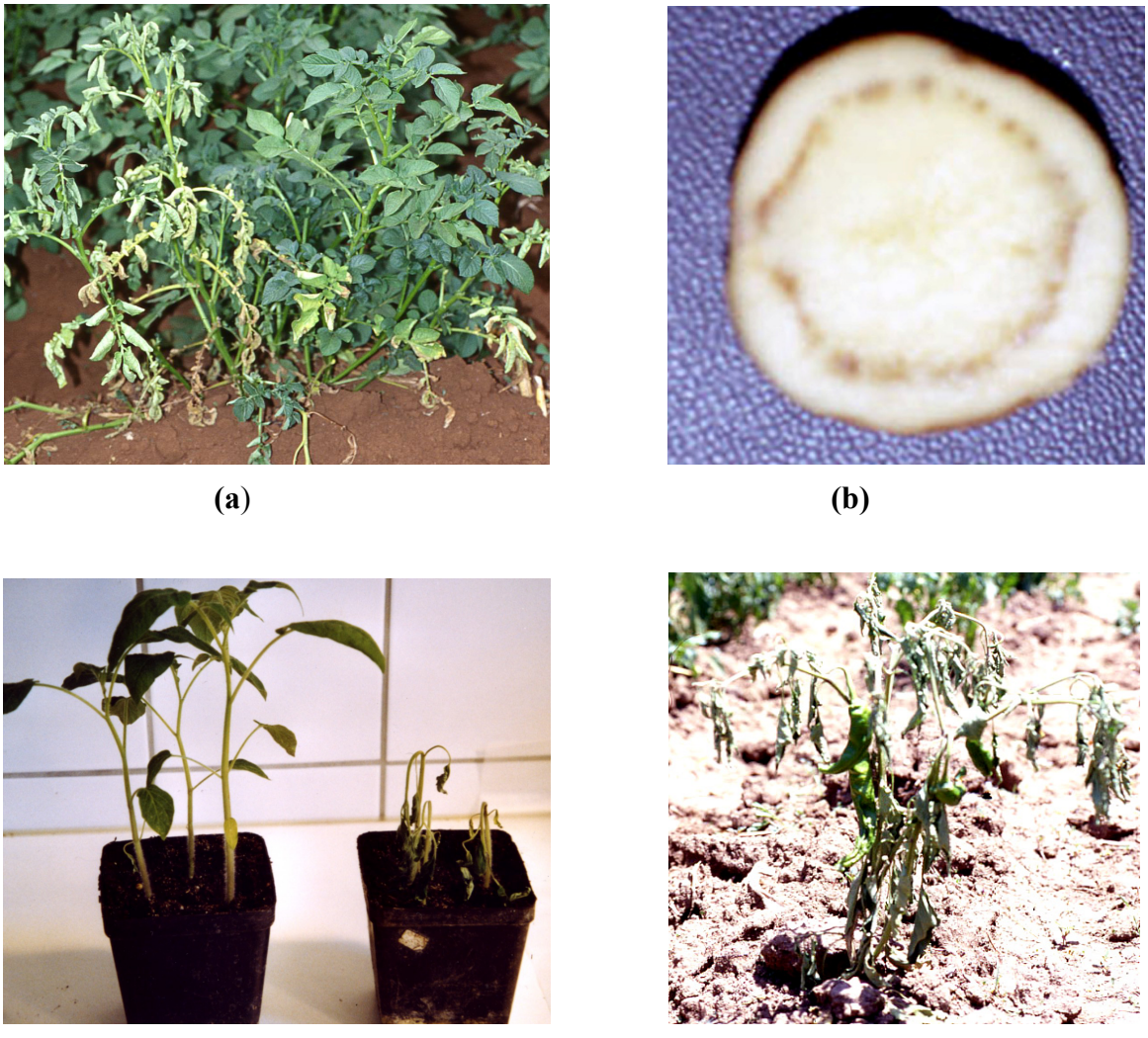
The host range of the bacterium is exceptionally wide, and many economically important crops as well as many weed hosts have been recognized (Hayward, 1991). It is a major constraint in the production of several important crops, particularly Solanaceae crops such as potato, tomato, eggplant, pepper, and tobacco (French and Sequeria, 1970). In addition to Solanaceae plants, leguminous plants (such as groundnut, French bean), a few monocotyledons (mainly banana, ginger) and several tree and shrub hosts (e.g. mulberry, olive, cassava, eucalyptus) are observed to be susceptible to the pathogen (Genin and Boucher, 2002). Generally, *R. solanacearum* has an extended host range that includes hundreds of plant species in 50 families of plants (Hayward, 1994). Recently, it was shown

that certain ecotypes of the model plant *Arabidopsis thaliana* are also susceptible to the pathogen (Deslandes *et al.,* 1998).

The pathogen is endemic in most subtropical and tropical regions of the world (Genin and Boucher, 2002). Nevertheless, in the 1990s, strains adapted to temperate climate (biovar II/race 3) were found in Western Europe on latently infected tubers and caused both economic and political problems when it appeared on potatoes (Janse, 1996). Despite ongoing eradication efforts, the bacterium is still present in a number of fields and waterways of Europe, although it has caused only minor direct crop losses (Swanson *et al.,* 2005). The strains (of biovar II) have been repeatedly introduced to Europe and North America in geraniums (*Pelargonium hortorum*) in recent years (Hamric, 2004; Janse *et al.,* 2004; Kim *et al.,* 2003; Williamson *et al.,* 2002) Moreover, it was among 10 plant pathogens listed in the Agricultural Bioterrorism Protection Act of 2002, so any finding of this organism in the United States is now subject to the strictest eradication requirements and the most stringent security regulations (Lambert, 2002).

The bacterium *R. solanacearum* is able to survive in the soil for long periods in the absence of host plants. Several explanations for this property have been proposed, such as the association of the bacteria with plant debris or with several weed hosts which are symptomless carriers (Hayward, 1991). For example, *R. solanacearum* strains collected in Europe can survive in water courses in roots of the weed *Solanum dulcamara* -bittersweet- (Elphinstone *et al.,* 1998). The long term survival of the bacterium could also be due to its ability to enter a dormant-like "viable but not culturable" state, like many other soil microbes (Grey and Steck, 2001). Moreover, the mode of invasion of *R. solanacearum* differs from that of most bacterial pathogens. It infects plants via root wounds or at sites of secondary root emergence, although aerial transmission by insects has also been reported for certain strains infecting

banana. Intercellular spaces of the root cortex and vascular parenchyma are sebsequently colonized and cell walls are disrupted, facilitating spread through the vascular system (Vasse *et al.,* 1995; Genin and Boucher, 2004). In xylem vessels, bacterial populations rapidly reach very high levels (>10^{10} cells/cm of stem in tomato), concomitant with wilting and plant death. The bacterium then returns to the soil, living as a saprophytic organism until it infects new host plant.



(a) **(b)**

(c) **(d)**

Fig. 1.1. Typical symptom of *Ralstonia solanacearum* disease on potato plant (a) and tuber (b), tomato (c) and pepper (d) plants

In Ethiopia bacterial wilt has been recorded on potato, tomato and eggplant in many regions (Yaynu, 1989). Stewart (1956) first recorded the disease in 1956 on potato and eggplant in Keffa region (south west Ethiopia). Stewart and Dagnachew (1967), in their index of plant diseases in Ethiopia, listed bacterial wilt on potato, tomato, and eggplant in Keffa (south Ethiopia) and on potato in Shewa and Arsi regions (central Ethiopia). Other workers observed bacterial wilt on potato and tomato crops in Ziway (south Ethiopia) and on potato in Ambo, Bako, and Guder (central Ethiopia) (SPL, 1981). Moreover, Yaynu (1989) indicated that bacterial wilt is an important disease of potato and tomato in many parts of Ethiopia and some time in the past the disease caused heavy losses at some commercial farms including at the potato seed tuber multiplication farm, Tseday Farm in Central Ethiopia, as a result of which potato seed tuber multiplication in the farm has been abandoned. Furthermore, per cent incidence as high as 45% (Yaynu and Korobko, 1986) and 63% (Bekele, 1996) on potato and 55% (EARO, 2002) on tomato were recorded in major potato producing areas of Ethiopia, giving good indication of the losses the disease can cause. Moreover, pepper plants infected by *R. solanacearum* have been observed in Ethiopia since recently. Its importance is increasing from time to time because of latently infected seed potatoes and decreasing land holdings that limit crop rotation (Berga *et al.,* 2000).

To date, no effective control methods exist for bacterial wilt disease. Plant breeding, field sanitation, crop rotation and use of bactericides have met, if at all, 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) coupled with the influence of environmental factors on host-pathogen interactions (Hayward, 1991; Hartman and Elphinstone, 1994) often restrict

the expression of resistance to specific regions (Thurstone, 1976). Thus, alternative control measures for the management of bacterial wilt caused by *R. solanacearum* need to be developed.

Biological control is proposed to be an effective, safe and ecologically friendly approach for plant disease management. Various recent studies have indicated that biological control of bacterial wilt disease could be achieved using antagonistic bacteria (Ciampi-Panno *et al.,* 1989; McLaughlin *et al.,* 1990). 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 much effective in the control 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, as a consequence of heterogeneity within the species *R. solanacearum* (Elphinstone, 1992), no single control method is likely to be universally effective. In order to select an effective control method for Ethiopian strains it is important to characterise *R. solanacearum* population from Ethiopia. Therefore, the central aim of this study is to characterise Ethiopian *R. solanacearum* strains and screening bacterial antagonists for the biological control of Ethiopian *R. solanacearum* strains.

Objectives of the study

1. To study the cultural and biochemical characteristics of Ethiopian *R. solanacearum* strains and identify biovars of *R. solanacearum* in Ethiopia
2. To investigate the pathogenic characteristics of Ethiopian *R. solanacearum* strains and designate the available races in Ethiopia
3. To study the genetic diversity of Ethiopian *R. solanacearum* strains and know the variation among the strains
4. To screen bacterial antagonists against *R. solanacearum* of Ethiopia and select effective antagonists for biological control

In order to meet the above specific objectives, a series of experiments were conducted at the Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute for Biological Control, Darmstadt, Germany, Max-Delbrück Centre (MDC) for Molecular Medicine, Berlin, Germany, and International University Bremen, School of Engineering and Science, Bremen, Germany, under the frame work of a PhD study sponsored by Catholic Academic Exchange Service (KAAD) scholarship.

2 Cultural and Biochemical Characterization of Strains of *Ralstonia solanacearum* from Ethiopia

Abstract

Ralstonia solanacearum (Smith) is a very destructive pathogen that causes wilt in potato, tomato and other solanaceae crops in Ethiopia. In order to study its cultural and biochemical characteristics and determine the types of biovars in Ethiopia, strains were isolated from different hosts from 18 localities in Ethiopia. The strains were confirmed to be *R. solanacearum* by growing on triphenyl tetrazolium chloride (TTC) medium, tomato bioassay and subjecting to a polymerase chain reaction (PCR) designed to amplify a single fragement (281 bp) of *R. solanacearum*. A total of sixty two strains were confirmed and characterized culturally and biochemically according to Hayward's classification scheme - based on their capacity to oxidize 3 disacchrides (lactose, maltose, and cellobiose) and 3 hexose alcohols (mannitol, sorbitol, and dulcitol). Moreover, in a separate study, the effect of temperature regime on static growth of *R. solanacearum* biovars was studied. The results of this study indicated that all strains from Ethiopia produce fluidal and irregular colonies with red center and whitish pheriphery on TTC medium and irregular, fluidal, creamy white colony on casamino acids-pepton-glucose (CPG) agar medium. On the basis of Hayward's classification method, 19 strains were placed in biovar I and 43 in biovar II. This is the first report of biovar I in Ethiopia. In the study on effect of temperature on *R. solanacearum* biovars, biovar II strains had significantly higher absorbance value than biovar I and III at cool temperature, while at higher temperatures the absorbance value of biovar I and III was significantly higher than that of biovar II.

2.1. Introduction

Ralstonia solanacearum (synonyms: *Pseudomonas solanacearum*) (Smith) (Yabucchi *et al.,* 1995) is a widespread and economically important bacterial plant pathogen (Hayward, 1991; Horita and Tsuchiya, 2001). It causes bacterial wilt, a major disease that limits production of diverse crops such as potato, tomato, egg plant, pepper, tobacco, banana and peanut (French and Sequeria, 1970; Williamson *et al.,* 2002). The pathogen has an extended host range that includes hundreds of plant species in 50 families (Hayward 1995).

In Ethiopia *R. solanacearum* is one of the most important and widely spreading bacterial disease of crops. Yaynu (1989) indicated that bacterial wilt is an important disease threatening the production of potato and tomato in many regions of Ethiopia. Moreover, incidence of the disease has been increasing from time to time and percent incidence as high as 45% (Yaynu and Korob, 1986) and 63% (Bekele, 1996) on potato and 55% (EARO, 2002) on tomato were recorded in major potato producing areas of Ethiopia.

The pathogen has been divided into different biovars on the basis of biochemical characteristics in culture (Hayward, 1964; Hayward *et al.,* 1990; He *et al.,* 1983). Within each of this biovars, there are numerous subtypes that may be associated with particular geographical locations (Buddenhagen and Kelman, 1964). In a previous study, 58 strains of *R. solanacearum* from potato and tomato collected from different localities of Ethiopia, were grouped in biovar II (Yaynu, 1989). Biovar II was reported to affect potatoes and tomatoes and rarely eggplant (French and Sequeira, 1970). However, in Ethiopia currently pepper plants are observed to be attacked by the pathogen and the intensity of the disease in Ethiopia is increasing from time to time. This may be due to introduction of new strains to Ethiopia from other parts of the world through latently infected planting material. Therefore, the aim of

this study was to characterise the strains collected from different locations in Ethiopia from potato, tomato, and pepper plants based on cultural and biochemical characteristics and determine biovars of *R. solanacearum* in Ethiopia.

2.2. Materials and Methods

2.2.1. Origin and collection of strains

Primarily eighty-one isolates were isolated from potato, tomato and pepper plants and potato tubers from various locations of Ethiopia (Fig. 2.1) where *R. solanacearum* is known to occur during 2003 cropping season from March to May. The altitude of the locations ranged from 1630 to 2600 meters above sea level. Additional six strains were obtained from the collection maintained by the Ethiopian Agricultural Research Organization (EARO) at the Plant Protection Research Centre (PPRC), Ambo, Ethiopia. Moreover, five strains were procured from Göttingen Collection of Phytopathogenic Bacteria, Institute for Plant Pathology and Plant Protection, Göttingen University, Germany, for comparison.

Strains from Ethiopian fields were isolated from diseased plants showing typical symptom of *R. solanacearum* from potato, tomato and pepper roots and from potato tubers in the Plant Protection laboratory of Jimma University College of Agriculture and Veterinary Medicine (JUCAVM), Jimma, Ethiopia. For the isolation from diseased plant roots, rootlets were thoroughly washed in tap water, and surface sterilized by dipping into 70% ethanol and flaming on Bunsel burner (OEPP/EPPO, 1990). Then they were chopped into 1 cm pieces into sterilized water in sterile capped bottle. On the other hand, for isolation from potato tubers, tubers were washed in tap water, surface sterilized by dipping into 70% alcohol and flaming on Bunsel burner and the epidermis around the heel end were removed using a regularly

disinfected scalpel. Then small tissue cores (diameter 5-10 mm, length 5 mm) removed from the heel ends were put in sterile water in sterilized bottle. The rootlets and tuber pieces were maintained in the water for 30 min (Wullings *et al.,* 1998) to diffuse the bacteria into the water. After 30 min 2 loopfuls of the water suspension was streaked on triphenyl tetrazolium chloride (TTC) medium (Kelman, 1954) containing 10 g pepton; 10 g dextrose; 1 g casamino acids (Difco), 18 g agar, 1 l distilled water, and filter sterilized 1% aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride, to give a final concentration of 0.005%, and incubated at 30°C for 48 h. After 48 h incubation, purification of *R. solanacearum*-looking colony was made and strains were temporarily maintained in distilled water until transportation to Germany for further study.

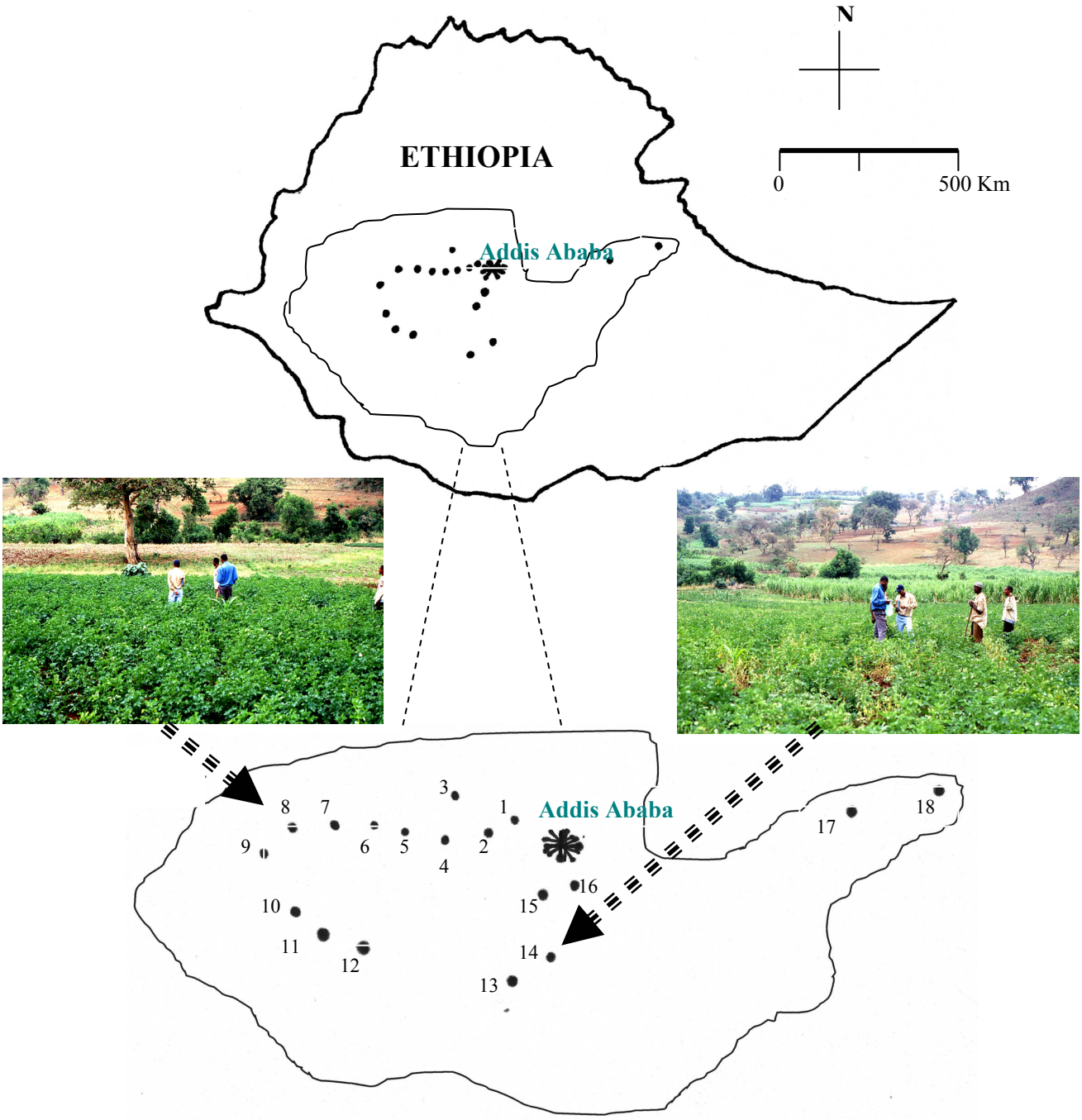


Fig. 2.1. Map of Ethiopia showing the major potato producing area and 18 localities from where collection of *Ralstonia solanacearum* was performed. 1 - Holeta; 2 - Ginchi, 3 - Jeldu, 4 - Ambo, 5 - Guder, 6 - Gedo, 7 - Bako, 8 - Kejo, 9 - Arjo, 10 - Agaro, 11- Jimma, 12 - Dedo, 13 - Awassa, 14 - Shashemene, 15 - Ziwa, 16 - Adami Tulu, 17 - Qarsa, 18 - Kombolcha, * - Capital city

2.2.2. Identification of *R. solanacearum* strains

Worldwide the most commonly used method for detection and identification of *R. solanacearum* has been isolation on TTC medium (Kelman, 1954) because of the relatively low cost, simplicity of use and consistency of results between different laboratories. Tomato bioassay is also currently recommended by the European Plant Protection Organization (EPPO) for detection of *R. solanacearum* in soil and potato tubers and for pathogenicity testing (Elphinstone *et al.,* 1996). Increasingly, other methods of identification using molecular methods like PCR are being used in commercial laboratories where speed and accuracy of diagnose are often important. With the development of *R. solanacearum* specific PCR methods, Ito *et al.* (1998) and Opina *et al.* (1997) were able to detect viable cells of *R. solanacearum.* In our study we combined the three methods so that the advantages of each method could be utilized.

TTC medium: During this test all isolates from Ethiopia and those from Göttingen were cultured on Kelman's TTC medium on replicated plates and incubated at 28°C. Identification of presumptive *R. solanacearum* colonies were made when typical colonies showed a characteristic red center and whitish periphery on TTC medium as described by Kelman (1954).

Tomato bioassay: Bioassay on tomato seedlings was aaccording to Janse (1988). All test strains which had presumptive *R. solanacearum* colony appearance on TTC medium were inoculated on two weeks old tomato seedlings (cv. Matina) grown in 12 cm pots. Three seedlings in replicate were inoculated with suspension of the isolates at the rate of 10^9 cfu/ml with punctures made with a sterile needle in the stem between the two cotyledons. Three replicates per bacterial suspension were used so that a total of nine seedlings were inoculated

with each bacterial concentration. Seedlings inoculated with sterile water were used as a control. Seedlings were held at 25°C in greenhouse and development of typical wilting symptom was recorded weekly. When typical symptoms were observed re-isolation of the bacteria was made on TTC to confirm the bacteria (Koch's rule). In symptomless plants, stem segements from the middle of the stem were washed and surface-disinfected with ethanol and imprinted five times with the cut ends of the stem on to the TTC medium (Prior *et al.,*1996). After 48 h incubation at 28°C, presence of *R. solanacerum*-looking colony was checked to know whether the pathogen was present in the plant latently or not.

Polymerase chain reaction (PCR): All strains that produced typical symptom in tomato assay and typical *R. solanacearum* colonies on TTC agar subsequentely were subjected to PCR for confirmation. Extraction of total genomic DNA from bacterial cells was performed using the "DNeasy Tissue Handbook" kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For PCR amplification a DNA thermal cycler (PeQlab Cyclone 25) was used. Reaction volumes were 50 µl and contained 1xPCR buffer (10 mM Tris HCl [pH 8.3], 50mM KHcl), 1.5 mM $MgCl_2$, 0.05mM of each dNTP, 25 pmol of primers 759 (5'GTCGCCGTCAACTCACTTTCC3') and 760 (5'GTCGCCGTCAGCAATGCGGAATCG3'), 1µl of genomic template DNA and 0.5 U of Taq DNA polymerase (Metabion, Martinsried, Germany). Samples were denaturated at 94°C for 3 min, annealed at 53°C for 1 min and extended at 72°C for 1.5 min, followed by 30 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 15 s, and a final extension of 72°C for 5 min (Opina *et al.,* 1997). PCR products were separated in 1.5% agarose gels, stained with ethidium bromide at 0.5µg/ml and visualized and photographed under ultraviolet (302 nm) light.

Table 2.1. List of *Ralstonia solanacearum* strains used for this study

Number	Strain designation	Host plant	Geographical origin	Altitude (m)
1	Pot 1	Potato	Mutulu, Guder, Ethiopia	2000
2	Pot 5	"	Mutulu, Guder, Ethiopia	2000
3	Pot 34	"	Jato, Gedo, Ethiopia	1700
4	Pot 4II	"	Tsedey, Holeta, Ethiopia	2400
5	Pot 5II	"	Tsedey, Holeta, Ethiopia	2400
6	Pot 9II	"	Tsedey, Holeta, Ethiopia	2400
7	Pot 10II	"	Tsedey, Holeta, Ethiopia	2400
8	Pot 15II	"	Mutulu, Guder, Ethiopia	2000
9	Pot 2JU	"	Jimma, Ethiopia	1700
10	Pot 4JU	"	Jimma, Ethiopia	1700
11	Pot 6JU	"	Jimma, Ethiopia	1700
12	Pot 8JU	"	Jimma, Ethiopia	1700
13	Pot 9JU	"	Jimma, Ethiopia	1700
14	Pot 6III	"	Kejo, Ethiopia	1650
15	Pot 10III	"	Kejo, Ethiopia	1650
16	Pot 16III	"	Bako, Ethiopia	1650
17	Pot 17III	"	Bako, Ethiopia	1650
18	Pot 20III	"	Arjo, Ethiopia	2350
19	Pot 21III	"	Arjo, Ethiopia	2350
20	Pot 29JU	"	Jimma, Ethiopia	1700
21	Pot 31JU	"	Jimma, Ethiopia	1700
22	Pot 40	"	Ginchi, Ethiopia	2200
23	Pot 42	"	Jeldu, Ethiopia	2600
24	Pot 46	"	Jeldu, Ethiopia	2600
25	Pot 48	"	Ginchi, Ethiopia	2200
26	Pot 50	"	Ginchi, Ethiopia	2200
27	Pot 55	"	Karafildicha, Shashemene, Ethiopia	1900
28	Pot 56	"	Karafildicha, Shashemene, Ethiopia	1900
29	Pot 57	"	Karafildicha, Shashemene, Ethiopia	1900
30	Pot 58	"	Karafildicha, Shashemene, Ethiopia	1900
31	Pot 59	"	Karafildicha, Shashemene, Ethiopia	1900
32	Pot 60	"	Karafildicha, Shashemene, Ethiopia	1900

Continued on following page

Number	Strain designation	Host plant	Geographical origin	Altitude (m)
33	Pot 61	Potato	Awassa, Ethiopia	1800
34	Pot 62	"	Awassa, Ethiopia	1800
35	Pot 65	"	Dedo, Jimma, Ethiopia	2200
36	Pot 66	"	Dedo, Jimma, Ethiopia	2200
37	Pot 68	"	Dedo, Jimma, Ethiopia	2200
38	Pot 70	"	Dedo, Jimma, Ethiopia	2200
39	Pot 71	"	Dedo, Jimma, Ethiopia	2200
40	Pot 81	"	Kombolcha, east Hararge, Ethiopia	2000
41	Pot 84	"	Ambo, Ethiopia	2600
42	Pot 86	"	Kejo, Ethiopia	1650
43	Pot 91	"	Karafildicha, Shashemene, Ethiopia	1900
44	Pot 92	"	Karafildicha, Shashemene, Ethiopia	1900
45	Pot 93	"	Karafildicha, Shashemene, Ethiopia	1925
46	Pot 94	"	Karafildicha, Shashemene, Ethiopia	1900
47	Tom 3	Tomato	Mutulu, Guder, Ethiopia	2000
48	Tom 1II	"	Tseday, Holeta, Ethiopia	2400
49	Tom 6II	"	Tsedey, Holeta, Ethiopia	2400
50	Tom 53	"	Karafildicha, Shashemene, Ethiopia	1900
51	Tom 56	"	Adam Tulu, Ethiopia	1600
52	Tom 58	"	Mutulu, Guder, Ethiopia	2000
53	Tom 88	"	Ziway, Ethiopia	1850
54	Pep 7	Pepper	Mutulu, Guder, Ethiopia	2000
55	Pep 58	„	Mutulu, Guder, Ethiopia	2000
56	Pep 61	„	Mutulu, Guder, Ethiopia	2000
57	Pot 1076PPRC*	Potato	Kombolcha, east Hararge, Ethiopia	2000
58	Pot 1079PPRC*	"	Qarsa, east Hararge, Ethiopia	----
59	Pot 262APPRC*	"	Goromt, Ambo, Ethiopia	2600
60	Pot 1080PPRC*	"	Qarsa, east Hararge, Ethiopia	2000
61	Pot 1091PPRC*	"	Agaro, Jimma, Ethiopia	1650
62	Tom 768PPRC*	Tomato	Arata Chaffa, Ziway, Ethiopia	1850
63	GSPB 2690**	Pepper	Kenya	__
64	GSPB 2695**	Tomato	Kenya	__
65	GSPB 2709**	Potato	Peru	2000
66	GSPB 2791**	"	Peru	__
67	GSPB 2792**	"	Cameroon	__

All the strains were collections of this study except those with * which were obtained from Plant Protection Research Center (PPRC), Ambo, Ethiopia, and those with ** which were procured from Göttingen Collection of Phytopathogenic Bacteria (GSPB),Göttingen, Germany.

2.2.3. Culturing and maintenance of cultures

All strains that were confirmed to be *R. solanacearum* with the methods mentioned before were streaked on TTC medium. After 48 h incubation at 30°C, wild type colonies of each isolate were selected on the basis of their fluidity, color, and morphology and stored in sterile distilled water in test tubes at room temperature (Kelman and Person, 1961; Wullings *et al.,* 1998) to reduce mutation (Lozano and Sequeira, 1970). To be used in different experiments, the strains were routinely cultured on tetrazolium chloride (TTC) medium (Kelman, 1954) and on casamino acids, peptone and glucose (CPG) agar (0.1% peptone, 0.01% casamino acids (Difco), 0.05% glucose, 1.5% (w/v) agar (Smith *et al.,* 1995) at 30°C.

2.2.4. Cultural characteristics

Strains were cultured on TTC and CPG medium at 30°C for 48 h and their shape and colour were noted for comparison. Fluorescence pigmentation was tested on King's B medium (King *et al.,* 1954) containing proetose peptone (Difco) 20 g, K_2HPO_4 .$3H_2O$ 2.5 g, $MgSO_4$. $7H_2O$ 6.0 g, agar (Difco) 15 g, glycerol 15 ml, and distilled water 1 l. Strains were incubated on this medium at 30°C for 48 h and after 48 h colonies were examined in the dark for fluorescence with a UV lamp (366 nm) according to Sands (1990).

2.2.5. Biochemical characteristics

Oxidase test: Oxidase activity was detected by the method of Kovacs (1956). Freshly grown (24 to 48 h) cultures from nutrient agar with 1% glucose were patched onto a filter paper moistened with a fresh oxidase reagent (1% w/v aqueous solution of tetramethyl-para-

phenylene diamine dihydrochloride) using a wooden stick. A purple reaction in 30 s was recorded as oxidase positive (Sands, 1990).

Catalase test: Catalase test was performed according to methods described by He *et al.* (1983). One ml of a 3% solution of hydrogen peroxide was added to a Petri dish and a loop of fresh culture grown on CPG agar medium was added into the solution. Release of bubble from the culture was recorded as catalase positive (Sands, 1990).

Nitrate reduction test: This test was accomplished as suggested by Hayward (1964) using the medium of Fahy and Hayward (1983). It was a semi-soft agar medium containing (g/l): pepton 10 g, Nacl 5 g, KNO_3 2 g, and agar 3 g, which was boiled to dissolve the agar. The pH was adjusted to 7.0 with concentrated NaOH, and the medium dispensed into test tubes and autoclaved. Tubes were stab inoculated with a loop of a test strain and then filled with sterile melted 3% water agar. Control test tubes were not inoculated with strains. Good growth in 5 days at 30°C was taken as indicative of nitrate reduction into nitrite (Sands, 1990).

KOH solubility test: The KOH solubility test was performed according Fahy and Hayward (1983) using 24 to 48 h culture. Two to three drops of 3% KOH were put onto glass slide and the colony of test strain was stirred into the solution with clean loop for 5 to 10 s . When the solution was viscous enough to stick to the loop causing a thin strand of slime, then the test is recorded as positive (KOH soluble).

NaCl tolerance test: For this test NaCl broth of Hayward (1964) was used which contained: pepton 5 g, yeast extract 3 g, glucose 5 g, distilled water 1 l, and either 5, 10, 15 or 20 g of Nacl, pH 7-7.2. The broth was autoclaved at 121°C for 15 min and dispensed into sterile 100

ml of flasks. Test strains were inoculated into the flasks and incubated on rotary shaker at 30°C with 100 rpm up to 14 days. Growth was detected every 2 days for each test tube.

Tween 80 hydrolysis: Fatty acid esterase activity was tested by streaking the bacteria onto a nutrient agar medium containing calcium chloride and Tween 80, a polymer consisting of polyoxy-ethylene-sorbitanmonooleate (Sands, 1990). The medium contains: peptone, 10 g; $CaCl_2$ dihydrochloride, 0.1 g; NaCl, 5 g; agar, 15 g; distilled water, 1 l; with the pH adjusted to 7.4. Tween 80 was autoclaved separately and added with 10 ml/l and mixed before plating. Incubation was made at 30°C for up to 7 days (Fahy and Hayward, 1983). An opaque zone of crystals around a colony was recorded as positive reaction for hydrolysis of Tween 80 (Sands, 1990).

H_2S production: This was detected according to Sands (1990) by using a medium which constitutes (g/l): $NH_4H_2PO_4$ 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$.$7H_2O$ 0.2 g, NaCl 5 g, yeast extract 5 g, cysteine hydrochloride (anhydrous) 0.1 g, and dispensed in 5 ml aliquots into tubes and autoclaved. Lead acetate impregnated paper strips were prepared by dipping 5 mm strips into 5% lead acetate, dried and autoclaved. These were hung above inoculated media using cotton plugs and black discoloration of the lead sulphide was monitored as an indication for H_2S production.

Starch hydrolysis: Nutrient agar plates containing 0.2% soluble starch (w/v) were streaked by the test strains and incubated at 30°C until heavy growth occurred. Then plates were flooded with IKI solution (iodine, 1 g; potassium iodide, 2 g; distilled water, 100 ml). A clear zone around a colony was recorded as positive reaction (Sands, 1990).

Gelatin hydrolysis: For this test, nutrient agar with 0.4% (w/v) gelatin was poured into Petri dishes, cooled and dried over night. The following day strains were inoculated on to each plate and incubated at 30°C for 3 days. When good growth was observed, the plate surfaces were flooded with 5 ml of mercuric chloride solution ($HgCl_2$, 12 g; distilled water, 80 ml; concentrated HCl, 16 ml) (Sands, 1990). A clear zone surrounding bacterial growth indicates positive reaction for the test (Dickey and Kelman, 1988).

Growth at 37 and 41°C: To determine the growth at 37 or 41°C, strains were cultured in flasks for 3 days in CPG broth on rotary shaker at the different temperatures and growth was read by spectrophotometer (Sands, 1990).

Carbohydrate oxidation test: To test oxidation of sugars and sugar alcohols, the basal medium described by Hayward (1964) was used. The medium constitutes: $NH_4H_2PO_4$ 1.0 g, KCl 0.2 g, $MgSO_4.7H_2O$ 0.2g, bromothymol blue (1%w/v) 0.3 ml, agar 1.5 g, distilled water 1 l. The pH was adjusted to 7.1 with 40% (w/v) NaOH solution before adding the agar.

Lactose, maltose, cellobiose, fructose and sucrose solutions were filter-sterilized, while D-glucose, mannitol and sorbitol were autoclaved for 20 min as 10% (w/v) solutions (Hayward, 1964). Dulcitol was added directly to the basal medium, which was then autoclaved for 20 min. Five ml of each sugar and sugar alcohol solutions were added to 45 ml of molten cooled Hayward's basal medium and 10 ml volumes of the resulting amended medium were dispensed into test tubes (Hayward, 1964). Hayward's medium without sugar or sugar alcohol carbon source served as control.

A suspension of each strain grown on CPG for 48 h at 30°C was prepared by inoculating 300 µl of sterile water with a wire loopful of cells (Williamson *et al.,* 2002). The test tubes of

Hayward's medium were inoculated with 30µl of the prepared suspensions and incubated at 30°C and checked for acid production (yellow color) (Fig. 2.2) at various intervals for up to 5 weeks (Hayward, 1964; He *et al.,* 1983) .

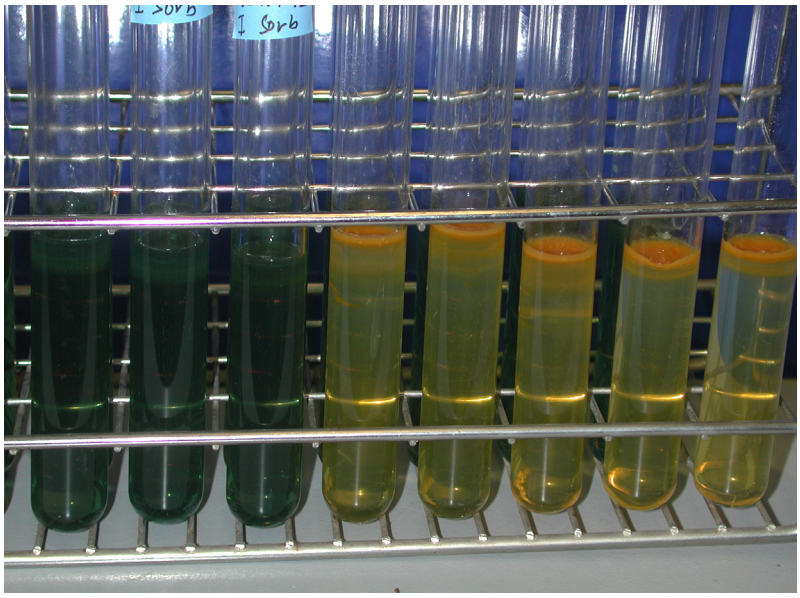


Fig. 2.2. Carbohydrate oxidation test for strains of *R. solanacearum.* Yellow color = + ve reaction; green color = - ve reaction.

2.2.6. Effect of temperature on static growth of *R. solanacearum*

In order to assess the effect of temperature on the growth of *R. solanacearum*, 10 strains from biovar I, 15 from biovar II, and 3 from biovar III were grown in static culture at 22, 27, 32 and 37°C. Growth in static culture was determined in 5 ml CPG broth in a water bath. Each bacterial suspension (OD 660 = 0.05) was prepared in duplicate test tubes and incubated at the four different temperature ranges. The absorbance after 48 h incubations was determined using a spectrophotometer (Spectronic 20 Genesys, Schütt Labortechnik, Göttingen, Germany). The experiment was repeated. Statistical analysis was performed to see the effect.

2.2.7. Statistical analysis

To determine single or interaction effects of factors (e.g., temperature and biovar), data were subjected to statistical analysis using the general linear model procedure (PROC GLM) of the SAS version 8 (SAS Institute, 1999). Since significant interactions were observed between factors, the level of one factor was compared at each level of the other factor. Significant factor effects were detected by means of ANOVA and treatment means at different levels of the respective factors were compared using Tukey's multiple means comparison procedure. A significant level of $\alpha = 0.05$ was used in the analysis.

2.3. Results

2.3.1. Identification

All the collected eighty-seven isolates from Ethiopia had relatively close colony appearance on TTC medium resembling *R. solanacearum*. However, when tomato bioassay was carried out by inoculating all the strains on tomato seedlings, 62 of the strains showed typical symptoms of wilting, whereas the remaining 19 were non-pathogenic and could be rated as saprophytic, although they had similar colony appearance with *R solanacearum*. Subsequently, when all the 62 strains that produced typical symptoms on tomato were subjected to a PCR using species specific primers 759 and 760, all produced a single 281 bp fragment (Fig. 2.3) which confirmed the identity of the 62 strains to be *R. solanacearum*. List of these strains is given in Table 2.1.

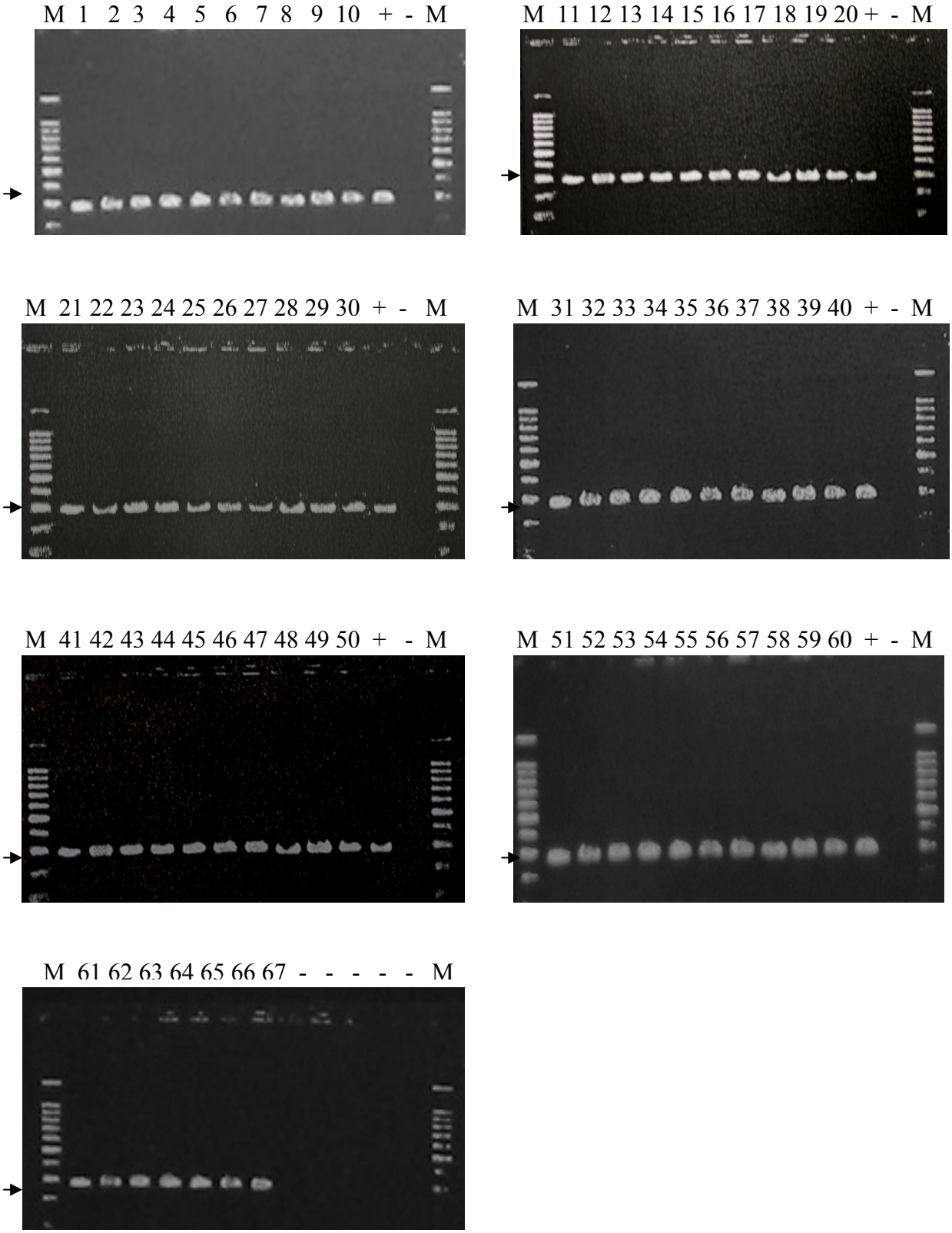


Fig. 2.3. Polymerase chain reaction (PCR) confirmation of *R. solanacearum* isolates collected from Ethiopia. The 281 bp PCR product amplified is visible on the ethidium bromide stained agarose gel. M= DNA marker; + = positive control (an identified *R. solanacearum* from Göttingen Phytobacteriology Collection); - = negative control (reaction mixture without template DNA); Lanes 1-10, 11-20, 21-30, 31-40, 51-60 and 61-62 are strains collected from Ethiopia; Lanes 63-67 are strains obtained from Göttingen Phytobacteriology Collection (i.e., GSPB 2690, GSPB 2695, GSPB 2709, GSPB 2791 and GSPB 2792).

2.3.2. Cultural characteristics

On TTC medium, all Ethiopian strains produced fluidal and irregular colonies with pink or light red center and whitish periphery after 48 h of incubation (Fig. 2.4a). However, when the strains lost their virulence by storage, the colony becomes smaller and round-shaped with deep red color. On another medium, CPG agar, all virulent strains produced irregular, smooth, creamy-white, and highly fluidal colonies which turned brown after 48 h of incubation (Fig. 2.4b). Strains of the Göttingen Collection of Phytopathogenic Bacteria, procured for comparison, also showed same colony appearance on both TTC and CPG medium. None of the strains produced fluorescent pigment on King's B medium.

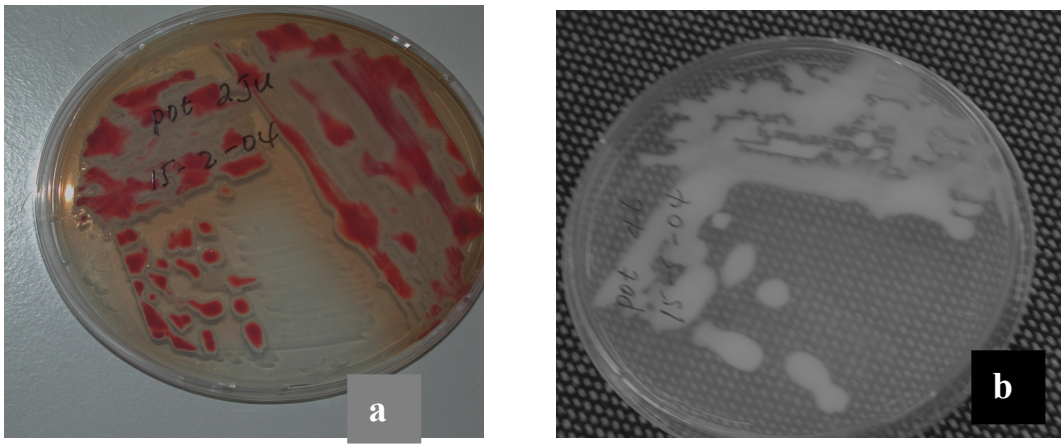


Fig. 2.4. Cultural characteristics of Ethiopian *Ralstonia solanacearum* strains on triphenyl tetrazolium chloride (TTC) (a) and casamino peptone glucose (CPG) (b) agar medium incubated at 30°C for 48 h.

2.3.3. Biochemical characteristics

Oxidase and catalase: All the 62 strains were oxidase positive; a purple colour appearing within 10 s after addition of culture to the oxidase reagent. They were all catalase positive; release gas upon addition of hydrogen peroxide (Table 2.2).

Nitrate reduction: All the confirmed strains reduced nitrate to nitrite. This was attested by good growth of the strains in Fahy and Hayward (1983) medium filled with 3% water agar in five days at 30°C.

KOH solubility: All the strains produced a thin strand of slime when drops of KOH were added on a glass slide and the colonies of the bacteria were lifted up with loop after stirring into the solution.

NaCl tolerance: In all the strains, heavy growth (turbidity) appeared in 0.5 and 1.0% NaCl medium with weak growth in 1.5% NaCl. As is the characteristics of *R. solanacearum,* none grew at 2% NaCl. However, when the bacteria from this 2% NaCl were streaked on a new salt free medium, a normal growth observed.

Starch, gelatine and Tween 80 hydrolysis: None of the strains hydrolysed starch or gelatine as no clear zone surrounding the bacterial growth was observed when the plates were flooded with IKI solution (for starch hydrolysis) or with mercuric chloride solution (for gelatine hydrolysis). However, all the strains hydrolysed Tween 80.

H_2S production: All the strains produced black discoloration on lead acetate impregnated paper strips hanged on medium of strains indicating production of H_2S.

Growth at 37 and 41°C: All strains grew at 37°C and all failed at 41^0C.

Table 2.2. Biochemical characteristics of strains of *Ralstonia solanacearum* from Ethiopia

Strain	Tests														Biovar
	Oxidase	Catalase	Nitrate reduction	KOH solubility	NaCl tolerance				Tween 80 hydrolysis	Starch hydrolysis	Gelatin hydrolysis	Growth at		H_2S production	
					0.5%	1%	1.5%	2%				37^0 C	41^0 C		
Pot 2JU	+[a]	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 4II	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 8JU	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 31JU	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 42	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 46	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 48	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 50	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 55	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 58	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 59	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 62	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 91	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 94	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Tom 6II	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Tom 53	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pep 7	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pep 58	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pep 61	+	+	+	+	+	+	+	-	+	-	-	+	-	+	I
Pot 1	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 5	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 34	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 5II	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 9II	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 10II	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 15II	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 4JU	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 6JU	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 8JU	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II

Continued on following page

Strain	Tests														Biovar
	Oxidase	Catalase	Nitrate reduction	KOH solubility	NaCl tolerance				Tween 80 hydrolysis	Starch hydrolysis	Gelatin hydrolysis	Growth at		H_2S production	
					0.5%	1%	1.5%	2%				37^0C	41^0C		
Pot 9JU	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 6III	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 10III	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 16III	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 17III	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 20III	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 21III	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 29JU	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 40	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 56	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 57	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 60	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 61	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 65	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 66	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 68	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 70	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 71	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 81	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 84	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 86	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 92	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 93	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Tom 3	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Tom 1II	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Tom 56	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Tom 58	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Tom 88	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II

Continued on following page

Strain	Tests														Biovar
	Oxidase	Catalase	Nitrate reduction	KOH solubility	NaCl tolerance				Tween 80 hydrolysis	Starch hydrolysis	Gelatin hydrolysis	Growth at		H_2S production	
					0.5%	1%	1.5%	2%				37^0C	41^0C		
Pot 1076PPRC	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 1079PPRC	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 262APPRC	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 1080PPRC	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Pot 1091PPRC	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
Tom 768PPRC	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
GSPB 2695	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
GSPB 2791	+	+	+	+	+	+	+	-	+	-	-	+	-	+	II
GSPB 2690	+	+	+	+	+	+	+	-	+	-	-	+	-	+	III
GSPB 2709	+	+	+	+	+	+	+	-	+	-	-	+	-	+	III
GSPB 2792	+	+	+	+	+	+	+	-	+	-	-	+	-	+	III

+ = positive reaction or growth, - = negative reaction or growth

Carbohydrate oxidation: All tested strains utilized glucose, fructose and sucrose invariably. However, marked differences were observed in the ability of the strains to oxidize three disaccharides (lactose, maltose and cellobiose) and three sugar alcohols (mannitol, sorbitol and dulcitol). Based on Hayward's classification scheme (Hayward, 1964), 19 of 62 strains were classified as biovar I and 43 as biovar II (Table 2.3). Biovar II strains produced acid from lactose, maltose and celllobiose but failed to oxidize mannitol, sorbitol and dulcitol, while biovar I strains oxidized none of the disaccharides and sugar alcohols even after 5 weeks of incubation. Biovar III strains from Göttingen collection oxidized all of the sugar and alcohol carbohydrates. Where there is a positive oxidation, acid production from the carbohydrates at 30°C was in 2-6 days except from fructose which took 6-14 days. No reaction was produced in inoculated media without a carbohydrate source.

Table 2. 3. Oxidation of carbohydrates by strains of *Ralstonia solanacearum* from Ethiopia

Strain	Carbohydrate									Biovar
	Glucose	Fructose	Sucrose	Lactose	Maltose	Cellobiose	Dulcitol	Mannitol	Sorbitol	
Pot 2JU	+ (1) [a]	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 4II	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 8JU	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 31JU	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 42	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 46	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 48	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 50	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 55	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 58	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 59	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 62	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 91	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 94	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Tom 6II	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Tom 53	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pep 7	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pep 58	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pep 61	+ (1)	+ (2)	+ (1)	-	-	-	-	-	-	I
Pot 1	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 5	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 34	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 5II	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 9II	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 10II	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 15II	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 4JU	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 6JU	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 8JU	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 9JU	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 6III	+ (1)	+ (2)	+ (1)	+ (2)	+ (1)	+ (1)	-	-	-	II
Pot 10III	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 16III	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 17III	+ (1)	+ (2)	+ (1)	+ (1)	+ (2)	+ (1)	-	-	-	II
Pot 20III	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 21III	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 29JU	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 40	+ (1)	+ (2)	+ (1)	+ (1)	+ (2)	+ (1)	-	-	-	II
Pot 56	+ (1)	+ (2)	+ (1)	+ (2)	+ (1)	+ (1)	-	-	-	II
Pot 57	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 60	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 61	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 65	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 66	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 68	+ (1)	+ (2)	+ (1)	+ (1)	+ (2)	+ (1)	-	-	-	II
Pot 70	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 71	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 81	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 84	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 86	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 93	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Tom 3	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Tom 1II	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Tom 56	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Tom 58	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Tom 88	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 1076PPRC	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 1079PPRC	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 262APPRC	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 1080PPRC	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Pot 1091PPRC	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
Tom 768PPRC	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
GSPB 2695	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
GSPB 2791	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	-	-	-	II
GSPB 2690	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	III
GSPB 2709	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	III
GSPB 2792	+ (1)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	+ (1)	III

[a] +(1) = positive reaction in 2-6 days; + (2) = positive reaction in 6-14 days; - = negative reaction

2.3.4. Effect of temperature on static growth of *R. solanacearum* biovars

The result indicated that temperature regime significantly (df = 3, F = 20.97, P < 0.0001) affected static growth of *R. solanacearum*. The two-way interaction between temperature and biovar was significant (df = 6, F = 20.86, P < 0.0001) indicating that the effect of temperature on growth of *R. solanacearum* is variable depending on the type of biovar. Accordingly, the average absorbance value of biovar I and biovar III at 22°C was significantly (df = 2, F = 17.98, P < 0.0001) lower than that of biovar II after 48 h, while at 27°C there was no significant difference between biovar I, biovar II and biovar III. On the other hand, at 32 and 37°C, the average absorbance value of biovar II was significantly lower than that of biovar I and II (Table 2.4).

When the effect of temperature on the static growth of strains was evaluated for each biovar separately, in biovar II absorbance value at 37°C was significantly lower than the absorbance value at 22, 27, and 32°C, while in biovar I and III, absorbance value at 22°C was significantly lower than the absorbance value at 27, 32 and 37°C (Table 2.4).

Table 2.4. Growth in static culture (absorbance, 660nm) of *Ralstonia solanacearum* biovar (I, II, and III) strains in casamino acids, peptone and glucose (CPG) medium for 48 h as affected by four different temperature regimes

Temperature (°C)	Biovar		
	I	II	III
22	0.311 B b	0.451 A a	0.280 B b
27	0.462 A a	0.471 A a	0.607 A a
32	0.564 A a	0.404 B a	0.617 A a
37	0.566 A a	0.299 B b	0.620 A a

Means within a row and column followed by the same upper case and lower case letters, respectively, are not significantly different (Tukey, α = 0.05).

2.4. Discussion

In the present study, identification of Ethiopian strains was made with different methods before characterization. It was found that, use of TTC medium alone as detection and identification method of *R. solanacearum* is less accurate, although it is less costly and simple. The study indicated that some saprophytic bacteria with closely related colony appearance to *R. solanacearum* could minimize the accuracy of TTC. However, tomato bioassay could be used as an identification scheme as recommended by the European Plant Protection Organization (EPPO) (Elphinstone *et al.,* 1996) as all the strains that produced typical symptom on tomato could be confirmed to be *R. solanacearum* with the most reliable method, PCR. With species specific primers (759 and 760) the PCR produced a single band of 281 bp from all of the 62 strains as reported by Opina *et al.* (1997) and Ito *et al.* (1998).

On the basis of cultural characteristics, all virulent *R. solanacearum* strains from Ethiopia resembled those strains from other regions of the world (He *et al.,* 1983; Kelman, 1954; Williamson *et al.,* 2002). They produce fluidal colonies with pink red center on TTC and fluidal creamy colony on CPG which is typical of the pathogen (Hayward, 1964; He *et al.,* 1983). Yaynu (1989) also reported the same result on strains from Ethiopia on TTC. On King's B medium no strain produced a fluorescent pigment. This confirms that *R. solanacearum* is not among the fluorescent species of bacteria (Hayward, 1960).

The capacity to oxidize sucrose is a stable characteristic of *R. solanacearum* (Hayward, 1964). Moreover, absence of growth at 2% NaCl is another characteristic of the pathogen (Ito *et al.,* 1998). In our study all the *R. solanacearum* strains were uniformly positive for sucrose oxidation and did not grow at 2% NaCl solution. Thus it could be concluded that all the 62

strains were *R. solanacearum* which is in agreement with the result of the confirmation study made by tomato bioassay and PCR in this study.

The biochemical tests demonstrated that the Ethiopian strains of *R. solanacearum* belong to biovar I and biovar II. Strains in biovar I were isolated from potato, tomato, and pepper, while strains in biovar II from potato and tomato. In previous studies from Ethiopia only biovar II which corresponds to race 3 has been reported from strains isolated from potato and tomato (Yaynu, 1989). Therefore, with this study biovar I of *R. solanacearum* is the first report from Ethiopia. Biovar I is the most widely distributed strain of *R. solanacearum* in the world (He *et al.,* 1983). Hence it is not surprising that biovar I is among the strains collected from Ethiopia, as several thousands of potato genotypes have been introduced to the country (Berga *et al.,* 1994) from the International Potato Center (CIP), Peru, and other parts of the world to develop high yielding and adaptable cultivars with resistance to the major stresses. Therefore, it is likely that new strains of *R solanacearum* have been introduced through latently infected planting material. It has been demonstrated that, *R. solanacearum* can be widely disseminated by vegetative propagating material (tomato, *Heliconia,* banana, and potato) and contaminated irrigation water and latently infected planting materials play a major role in its local and international dissemination (Hayward, 1991; Williamson *et al.,* 2002).

Until 1989, only potato, eggplant and tomato have been found to be infected by *R. solanacearum* in Ethiopia (Yaynu, 1989). Since recently, however, pepper plants have been found wilting by the pathogen (personal observation). Moreover, the disease incidence on potato (Bekele, 1996) and tomato (EARO, 2002) has been increasing. This increase in the intensity of the disease and host-range of the pathogen may be due to the introduction of new strains to the country although it is difficult to distinguish it from changes in the cultural practices which can also intensify the development of the disease. However, the identification

of a newly introduced biovar in the country is a clue for the probable introduction of new strains in biovar II as well. On the other hand, the number of strains of the new biovar is almost one third (19 out of 62) of the whole population of the strains collected. This can be an indication that the introduction of the new strains to Ethiopia may not be very recent rather some long years back. One justification for this is that some of the strains in the new biovar were collected from areas far away from locations (Holeta, Shashemene and Alemaya) where Ethiopia is mainly multiplying introduced potato genotype before distributing to farmers. In 1989 Yaynu (1989) reported the existence of only Biovar II in Ethiopian *R. solanacearum* population.

A static culture test showed that biovar II strains were able to grow to a fairly high density at low temperature (22°C) in contrast to biovar I and II strains (Table 2.4) which grew to a higher density at higher temperatures (32 and 37°C). Thus this study clearly indicated that biovar II strains prefer a relatively cool temperature than biovars I and III which preferred higher temperatures. This is in agreement with the assumptions that biovar II and biovars I and III strains are adapted to cool and warm tropical climates, respectively, (Horita *et al.,* 2005; Swanson *et al.,* 2005; OEPP/EPPO, 2004; French *et al.,* 1993; Marin and El-Nashaar, 1993).

Though the adaptation of biovar II strains is to cool tropical conditions, strains of biovar II in Ethiopia were collected from wide range of altitude ranging from 1600 to 2600 meters above sea level. Likewise, biovar I strains from Ethiopia were collected from areas ranging from 1630 to 2600 meters above sea level, unlike its adaptation to warm tropical conditions. Moreover, of the three biovar III strains considered in the study, one strain was from 2000 meters above sea level in Peru (origin of the other two strains was not recorded) as opposed to its adaptation to warm tropical conditions. This may indicate that the strains can occur and

cause disease even out of their area of adaptation. There are also some reports which indicate cases of infection by *R. solanacearum* strains out of their range of adaptation (French *et al.,* 1993) and it is believed to be due to the distribution of infected potato tubers from one area to the other.

3 Pathological Characterization of Strains of *Ralstonia solanacearum* from Ethiopia and Effect of Age of Inoculation on Susceptibility of Hosts against *R. solanacearum*

Abstract

Ralstonia solanacearum (Smith) is a diverse species that differs in host range and reaction on tobacco leaf and can be differentiated into races based on its host reaction. Sixty-two strains collected from different hosts of Ethiopian fields were pathogenically characterized by inoculating into five differential hosts (potato, tomato, eggplant, pepper and tobacco). Moreover, reaction of the strains on tobacco leaf was studied by infiltrating suspension of the pathogen into tobacco leaf. In a separate study, the effect of age of inoculation on susceptibility of differential hosts was studied by inoculating the differential hosts at 1-2, 3-4, and 5-6 true leaf stages, with race 3 and race 1 strains in a completely randomized design. The study showed that all the strains cause rapid wilt in potato, tomato and eggplant, while their reaction in pepper and tobacco is variable. On the basis of pathogenicity tests, 19 strains were grouped into race 1 and 43 strains into race 3. All the strains in race 3 caused yellow chlorosis on infiltrated tobacco leaf, while those in race 1 developed hypersensitivity reaction (HR) or necrosis. Race 1 has not been reported from Ethiopia and is a new report with this study. The presence of race 1 strains in Ethiopia raises concern as the bacterium has wide host range than race 2 strains. The effect of age of inoculation on wilt development depended on type of host and race of the pathogen. Generally, age of inoculation did not significantly affect wilt development in potato, tomato, and eggplant, while in pepper and tobacco plant inoculation at 5-6 true leave stages significantly reduced wilt development indicating the existence of age related resistance against the pathogen.

3.1. Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) (Yabucchi *et al.,* 1995) is one of the most important and widespread disease of numerous crops in tropical, subtropical and temperate regions of the world (Hayward, 1991). The host range of the pathogen is exceptionally wide, and many economically important crops as well as many weed hosts have been recognized (Hayward, 1991). The pathogen *R. solanacearum* is a diverse species that differs in host range, geographical distribution, pathogenicity and biochemical properties (Horita and Tsuchiya, 2001). In Ethiopia, *R. solanacearum* is one of the most important pathogens (Yaynu, 1989) threatening the production of potato and tomato in different parts of the country. The intensity of the disease is increasing from time to time and percent incidence as high as 45% (Yaynu and Korob, 1986) and 63% (Bekele, 1996) in potato and 55% (EARO, 2002) in tomato was recorded in major potato producing parts of Ethiopia

Strains of *R. solanacearum* are differentiated into five races according to host range (Buddenhagen *et al.,* 1962; He *et al.,* 1983; Pegg and Moffet, 1971) and five biovars according to utilization of three disaccharides and three hexose alcohols (Hayward, 1964; Hayward, 1994; He *et al.,* 1983). Buddenhagen *et al.* (1962) separated isolates into three races, differing mainly in host range, as follows: race 1, pathogenic to tobacco, tomato, and many other solanaceous plants, and certain diploid bananas; race 2, pathogenic to triploid bananas and *Heliconia* spp; race 3, pathogenic to potato and tomato, but only weakly pathogenic to other solanaceous crops. Later on He *et al.* (1983) designated strains from Mulberry as race 4, biovar IV.

In a previous study in Ethiopia, strains of *R. solanacearum* from potato and tomato from different localities of the country were grouped into biovar II (Yaynu, 1989) which

corresponds to race 3. This race 3 attacks only potato, tomato and rarely egg plant (French and Sequeira, 1970, Horita *et al.,* 2005). However, currently in Ethiopia besides potato and tomato, pepper plant was also observed to be attacked by the pathogen. This new infection needs investigations as it may be due to the introduction of new strains of *R. solanacearum* to Ethiopia. Berga *et al.* (1994) has indicated that Ethiopia was introducing potato tubers from different parts of the world mainly from the International Potato Center (CIP) in South America, Peru, to improve the productivity and resistance of the local germplasm. Therefore it is likely that this new observation could be due to the introduction of a new strain to Ethiopia via introduced tubers and planting materials. Hence it is important to make host range studies for Ethiopian *R. solanacearum* strains so that the available races can be known.

Race determinations in most research reports (e.g., He *et al.,* 1983; Hayward 1991; Horita and Tsuchiya, 2001) were made by inoculating the differential hosts at 3 to 4 true leave stages as a standard. However, inoculation of hosts may be done before or after this standard stage because of difference in growth conditions of the plants that can influence growth of the plants. Nevertheless, there were no reports on the effect of age of plant inoculation on the occurrence of the disease on hosts and thereby race determination. Hence, it is also important to know the effect of age of differential host inoculation on disease development.

Therefore, the objectives of this study were: firstly, to investigate the pathogenic characteristics of Ethiopian *R. solanacearum* strains collected from different hosts and determine their host ranges and races, and secondly, to assess the effect of age of plant inoculation on the development of bacterial wilt caused by *R. solanacearum.*

3.2. Materials and Methods

3.2.1. Pathogenicity tests

Plants: For pathogenicity tests the plants used were potato (*Solanum tuberosum* L. "Secura"), tomato (*Lycopersicon esculentum* Mill. "Matina"), eggplant (*Solanum melongena* L. "Lange Violete"), pepper (*Capsicum annum* L. "Neusiedler Ideal") and tobacco (*Nicotiana tabacum* L. "White Burley"). Seed of each plant species was sown in a plastic tray filled with a commercial potting medium which constitutes a mixture of sand and commercial potting substrate (Fruhstorfer Erde Typ LD 80; Industrie-Erdenwerk Archut, Lauterbach, Germany) in a 1 to 3 ratio, respectively. The substrate was composed of humus, clay, and peat in a proportion of 15:35:50, respectively, and has a high water holding capacity due to high proportion of peat. Plants were germinated in a greenhouse at 26 to 30°C and 60 to 80% relative humidity with 12 h light and 12 h dark with the light from sodium high pressure lamps. Seedlings were transplanted into plastic pots (12 cm × 12 cm) containing the same potting medium mentioned above and grown in a greenhouse at 26 to 30°C (Fig. 3.1). Seedlings were watered every other day.

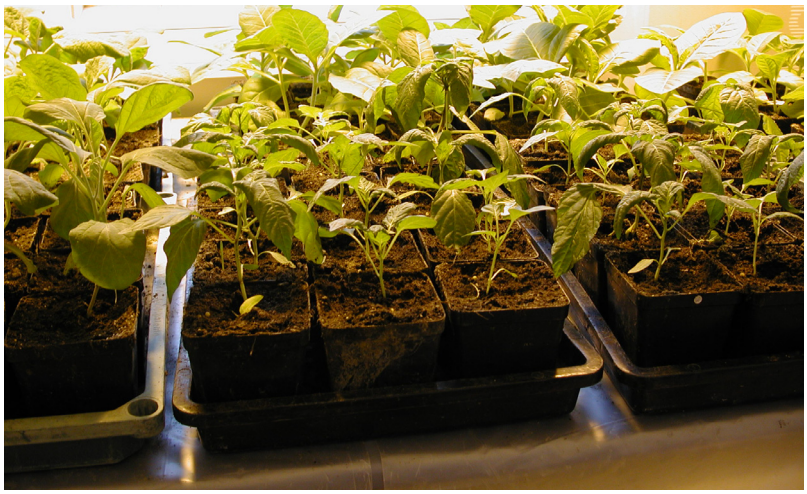


Fig. 3.1. Partial view of experimental set-up in greenhouse

Inoculation: For inoculation, all bacterial strains listed in Table 2.1 (Chapter 2) were used. The bacteria were grown on casamino acids, peptone and glucose (CPG) agar (Smith *et al.,* 1995) for two to three days at 30°C, suspended in sterile distilled water and adjusted to 10^8 to 10^9 cfu/ml. Inoculation was made at the three to four true leaf stage by puncturing the stem at the axils of the third fully expanded leaves from the apex (Fig.3.2) with a needle dipped in inoculum (Winstead and Kleman, 1952). Nine plants of each host were inoculated with each strain. Plants inoculated with sterile water served as negative control. Inoculated plants were maintained in greenhouse at 26 to 30°C with 12 h light and 12 h dark with the use of sodium high-pressure lamps during the light. Prior to inoculation, plants were not watered for 24 h (Williamsson *et al.,* 2002; OEPP/EPPO, 1990). The experiment was undertaken with completely randomized design with three replication and repeated.

Fig. 3.2. Inoculation of host plants at three to four true leaf stage by puncturing the stem at the axils of third fully expanded leaves with a needle dipped in inoculum

Severity of wilting was recorded at weekly intervals (He *et al.,* 1983; Horita and Tsuchiya, 2001) after inoculation on the following scale: 1 = no symptom, 2 = leaf above inoculation point wilted, 3 = two or three leaves wilted, 4 = four or more leaves wilted, and 5 = plant died.

3.2.2. Hypersensitivity reaction

To test the ability of strains to induce a hypersensitivity reaction (HR) on tobacco leaves, the method described by Lozano and Sequeira (1970) was adopted. Seeds of *Nicotiana tabacum* L. variety "White Burley" were sown on plastic tray filled with the potting medium similar to the pathogenicity test above and the emerging seedlings were grown for 30 days in greenhouse at 26 to 30°C temperature and 60 to 80% relative humidity. Each seedling was transplanted into a 12 cm × 12 cm pot containing a potting substrate mentioned above.

For tobacco leaf infiltration, all of the strains used for pathogenicity test were used. Each isolate was streaked on triphenyl tetrazolium chloride (TTC) medium (Kelman, 1954). After 48 h incubation at 30°C, wild type colonies of each isolate were selected on the basis of their fluidity, color and morphology.

When the tobacco plants were 30 to 45 days old (Lozano and Sequeira, 1970), from date of transplanting, the fully expanded leaves were infiltrated by injecting water suspension of bacteria into the intercellular spaces with a hypodermic syringe fitted with a fine needle (Klement *et al.,* 1964). From the lower side of the leaves, an area of 3-5 cm^2, in each of 8 to 12 intercostal areas of each leaf, was infiltrated with a suspension of 10^8 to 10^9 cfu/ml. At least 6 leaves were infiltrated by each isolate. Leaves infiltrated with sterile water served as a negative control. Leaf reactions were recorded at 24 and 48 h (Horita and Tsuchiya, 2001) incubation under the greenhouse conditions described above.

3.2.3. Influence of age of inoculation on the susceptibility of host plants against *R. solanacearum*

For observing whether age of inoculation of the host can affect susceptibility of hosts to *R. solanacearum*, a strain from race 1 (virulent to potato, tomato, eggplant, pepper and tobacco) and a strain from race 3 (virulent to potato, tomato and eggplant and non virulent to pepper and tobacco) were inoculated to all the test plants used for pathogenicity test at three different stages of the plants. The ages of inoculation were, 1 to 2, 3 to 4, and 5 to 6 true leaf stage. Method of inoculation, concentration of the inoculum and disease assessment were the same as in pathogenicity test. The experiment was carried out with completely randomized design with 3 replication and repeated.

For statistical analysis rated disease severity grades were converted to percentage severity index (PSI) with the formula described by Fininsa (2003) as :

$$PSI = S_{nr} \times 100/N_{pr} \times M_{sc},$$

where S_{nr} is the sum of numerical ratings, N_{pr} is the number of plants rated and M_{sc} is the maximum score on the scale.

3.2.4. Statistical analysis

Percentage severity values were statistically analysed using SAS version 8 (SAS Institute, 1999). Data of the disease repeated over time were checked for homogeneity of variance using the HOVTEST = LEVENE option of ANOVA procedure and pooled as variance homogeneity could be assumed. Single and interaction effects of factors (age, host and race)

were analysed by the general linear model (PROC GLM) procedure in SAS. Since significant interaction effects between factors were detected by ANOVA, treatment means of one factor were compared at each level of the other factor. Means were compared using the Tukey's mean comparison procedure. A significant level of $\alpha = 0.05$ was used in all analysis.

3.3. Results

3.3.1. Pathogenicity test

With the pathogenicity test, initial symptoms of wilting in susceptible hosts appeared 3-4 days after stem inoculation. Initial symptoms usually consisted of wilting of the inoculated leaf and stunting of growth. In potato, tomato and eggplants, inoculation was mostly followed by considerable ooze and decay of the pith surrounding the point of inoculation after which the plants wilted rapidly and died. All strains caused rapid wilting of eggplant, tomato and potato, while only 19 strains from Ethiopia and 3 strains from the Göttingen collection infected pepper and 15 strains from Ethiopia and 2 strains from Göttingen collection infected tobacco. Our observation showed that the progress of wilt in tobacco and pepper was not as rapid as in potato, tomato and eggplant.

On the basis of host reaction, the strains studied could be categorized into three pathogenic groups (Table 3.1). Group 1 included strains which were virulent on all five host species; group 2 included few strains which were virulent on all host species except tobacco; and group 3 included the majority of the strains which were virulent on potato, tomato and eggplant but non virulent on pepper and tobacco. Because of their wide host range, strains in group 1 and 2 were considered members of race 1, while those in group 3 were grouped into race 3 of Buddenhagen *et al.* (1962).

Table 3.1. Pathogenicity of strains of *Ralstonia solanacearum* from Ethiopia on five major hosts and their classification into pathogenic group and race

Strain	Original host	Pathogenicity rating[a]					Pathogenic group	Race	Biovar
		Potato	Tomato	Eggplant	Pepper	Tobacco			
Pot 2JU	Potato	H	H	H	M	M	1	1	I
Pot 4II	"	H	H	H	M	M	1	1	I
Pot 8 JU	"	M	H	M	M	L	1	1	I
Pot 31 JU	"	H	H	H	H	H	1	1	I
Pot 46	"	H	H	H	H	H	1	1	I
Pot 48	"	H	H	H	M	L	1	1	I
Pot 50	"	H	M	H	L	L	1	1	I
Pot 55	"	H	H	H	M	L	1	1	I
Pot 58	"	H	H	H	M	M	1	1	I
Pot 59	"	H	H	H	M	M	1	1	I
Pot 94	"	H	M	H	M	L	1	1	I
Tom 6II	Tomato	H	H	H	M	M	1	1	I
Tom 53	"	H	H	H	H	M	1	1	I
Pep 58	Pepper	H	H	H	M	L	1	1	I
Pep 61	"	H	H	H	M	L	1	1	I
GSPB 2690	"	H	H	H	M	L	1	1	III
GSPB 2709	Potato	H	H	H	H	M	1	1	III
GSPB 2792	"	H	H	H	M	M	1	1	III
Pot 42	"	H	H	M	M	0	2	1	I
Pot 62	"	M	H	H	M	0	2	1	I
Pot 91	"	H	H	H	M	0	2	1	I
Pep 7	Pepper	H	M	H	M	0	2	1	I
Pot 1	Potato	M	M	H	0	0	3	3	II
Pot 5	"	M	M	M	0	0	3	3	II
Pot 34	"	H	H	H	0	0	3	3	II
Pot 5 II	"	M	M	H	0	0	3	3	II
Pot 9II	"	H	M	H	0	0	3	3	II
Pot 10II	"	H	H	H	0	0	3	3	II
Pot 15II	"	M	M	M	0	0	3	3	II
Pot 4JU	"	H	H	H	0	0	3	3	II

Continued on next page

Strain	Original host	Pathogenicity rating[a]					Pathogenic group	Race	Biovar
		Potato	Tomato	Eggplant	Pepper	Tobacco			
Pot 6 JU	Potato	M	H	H	0	0	3	3	II
Pot 9JU	“	H	H	H	0	0	3	3	II
Pot 6III	“	H	H	H	0	0	3	3	II
Pot 10III	“	H	H	M	0	0	3	3	II
Pot 16III	“	M	M	H	0	0	3	3	II
Pot 17III	“	M	M	M	0	0	3	3	II
Pot 20III	“	M	M	H	0	0	3	3	II
Pot 21 III	“	H	M	H	0	0	3	3	II
Pot 29JU	“	M	M	M	0	0	3	3	II
Pot 40	“	H	H	H	0	0	3	3	II
Pot 56	“	H	M	M	0	0	3	3	II
Pot 57	“	M	M	M	0	0	3	3	II
Pot 60	“	M	M	M	0	0	3	3	II
Pot 61	“	H	H	H	0	0	3	3	II
Pot 65	“	H	H	H	0	0	3	3	II
Pot 66	“	M	M	H	0	0	3	3	II
Pot 68	“	H	H	H	0	0	3	3	II
Pot 70	“	M	H	H	0	0	3	3	II
Pot 71	“	M	M	M	0	0	3	3	II
Pot 81	“	H	H	H	0	0	3	3	II
Pot 84	“	M	M	M	0	0	3	3	II
Pot 86	“	M	M	H	0	0	3	3	II
Pot 92	“	H	M	H	0	0	3	3	II
Pot 93	“	H	H	H	0	0	3	3	II
Tom 3	Tomato	H	H	H	0	0	3	3	II
Tom 1II	“	M	M	M	0	0	3	3	II
Tom 56	“	M	H	H	0	0	3	3	II
Tom 58	“	H	H	H	0	0	3	3	II
Tom 88	“	H	H	H	0	0	3	3	II

Continued on next page

Strain	Original host	Pathogenicity rating[a]					Pathogenic group	Race	Biovar
		Potato	Tomato	Eggplant	Pepper	Tobacco			
Pot 1076PPRC	Potato	M	M	M	0	0	3	3	II
Pot 1079PPRC	"	M	M	M	0	0	3	3	II
Pot 262APPRC	"	H	H	H	0	0	3	3	II
Pot 1080PPRC	"	M	M	M	0	0	3	3	II
Pot 1091PPRC	"	M	M	H	0	0	3	3	II
Tom 768PPRC	Tomato	M	H	H	0	0	3	3	II
GSPB 2695	"	M	M	H	0	0	3	3	II
GSPB 2791	Potato	H	H	M	0	0	3	3	II

Results based on average disease indices of 9 plants 21 days after inoculation. H = high (4.1-5.0), M = medium (2.6-4), L = low (1.1-2.5), and 0 = no symptom (1.0).

3.3.2. Hypersensetivity reaction (HR)

Most of the strains in race 1 elicited slow spreading necrosis on infiltrated tissue typical of compatible interactions, and were pathogenic to tobacco. Tobacco leaves infiltrated with such strains induced a distinct yellow zone at the spreading edge of the lesion. The lesion became progressively darker, and the yellow halo surrounding the dark central area became more noticeable by 48 h (Fig. 3.3a). Gradually, the bacteria became systemic and there was extensive wilting, yellowing and necrosis of the leaf tissue. The exceptions in race 1 were strains Pot 7, Pot 42, Pot 62 and Pot 91 which induced a classic rapid HR on tobacco leaves (Fig. 3.3b). By 24 h they induced a reaction where in the leaf tissues bleached, and water soaked and the affected area was sharply delimited from the non-inoculated tissues by a definite border. Water was rapidly lost and the affected areas became thin, white and translucent by 60 h after infiltration. In contrast, all of the Ethiopian *R. solanacearum* strains in race 3 caused a yellowish discoloration of infiltrated tissue of tobacco after 48 h, but were non-pathogenic to tobacco (Fig. 3.3c). The degree of yellowing increased slowly, but the size of lesion remained constant and the bacteria did not invade adjoining tissues even after 4 days after infiltration. Tobacco leaves infiltrated with sterile water were unaffected. The reaction of tobacco leaves to all the strains under study was presented in Table 3.2.

Fig. 3. 3. Tobacco leaf reactions 48 h after infiltration with *Ralstonia solanacearum* suspensions of 1×10^{9} cfu/ml. (a) Dark brown necrotic reaction (N) induced by compatible (race 1) strains; (b) hypersensitivity reaction (HR) induced by non compatible (race 1) strains; (c) yellowish discoloration (Y) induced by non compatible (race 3) strains

Table 3. 2. Reaction of tobacco leaves to infiltration with strains of *Ralstonia solanacearum* from Ethiopia

	Hours after infiltration	
Strain	24	48
Pot 2JU	-[a]	N
Pot 4II	-	N
Pot 8 JU	-	N
Pot 31 JU	-	N
Pot 46	-	N
Pot 48	-	N
Pot 50	-	N
Pot 55	-	N
Pot 58	-	N
Pot 59	-	N
Pot 94	-	N
Tom 6II	-	N
Tom 53	-	N
Pep 58	-	N
Pep 61	-	N
GSPB 2690	-	N
GSPB 2709	-	N
GSPB 2792	-	N
Pot 42	HR	HR
Pot 62	HR	HR
Pot 91	HR	HR
Pep 7	HR	HR
Pot 1	-	C
Pot 5	-	C
Pot 34	-	C
Pot 5 II	-	C
Pot 9II	-	C
Pot 10II	-	C
Pot 15II	-	C
Pot 4JU	-	C
Pot 6 JU	-	C
Pot 9JU	-	C
Pot 6III	-	C
Pot 10III	-	C
Pot 16III	-	C
Pot 17III	-	C
Pot 20III	-	C
Pot 21 III	-	C
Pot 29JU	-	C
Pot 40	-	C
Pot 56	-	C
Pot 57	-	C
Pot 60	-	C
Pot 61	-	C
Pot 65	-	C
Pot 66	-	C
Pot 68	-	C
Pot 70	-	C
Pot 71	-	C
Pot 81	-	C
Pot 84	-	C
Pot 86	-	C
Pot 92	-	C
Pot 93	-	C
Tom 3	-	C
Tom 1II	-	C
Tom 56	-	C
Tom 58	-	C
Tom 88	-	C
Pot 1076PPRC	-	C
Pot 1079PPRC	-	C
Pot 262APPRC	-	C
Pot 1080PPRC	-	C
Pot 1091PPRC	-	C
Tom 768PPRC	-	C
GSPB 2695	-	C
GSPB 2791	-	C

[a] - = No reaction, N = slow or spreading necrosis, HR = hypersensitivity reaction, and C = slow collapse of infiltrated area

3.3.3. Influence of age of inoculation on the susceptiblity of host plants to *R. solanacearum*

There was significant three-way interaction between age, race and host (age*host*race: df = 8, $F = 28.14$, $P < 0.0001$) on the susceptibility of host plants to *R. solanacearum* strains (Table 3.3). As a result, the effect of age of inoculation on development of the disease on the hosts was analyzed separately for each host and race at respective age of inoculation.

Table 3.3. Summary of ANOVA results for effect of age of inoculation on the development of bacterial wilt caused by two races (race 1 and 3) of *Ralstonia solanacearum* on five different hosts (potato, tomato, eggplant, pepper and tobacco)

Source of variations	df	*F*	*P*
Age	2	97.52	<0.0001
Race	1	373.38	<0.0012
Host	4	533.37	<0.0001
Age*race	2	83.10	<0.0001
Age*host	8	25.37	<0.0001
Race*host	4	107.36	<0.0002
Age*race*host	8	28.14	<0.0001
Error	60	-	-

The effect of age of inoculation on disease severity depended on type of host and race. In potato, tomato and eggplant, there was no significant difference in disease index between all stages of inoculation for both races tested. However, in pepper and tobacco disease index was affected by age of inoculation and the effect depended on type of race. Accordingly, disease at

5-6 true leave stage was significantly lower than the disease index at 1-2 and 3-4 true leave stage for race 1, but for race 3 no significant difference was observed between all stages in disease development. Moreover, at a given stage of inoculation, there was no significant difference in disease index between the two races in potato, tomato and eggplant (Fig. 3.4 a,b,c), while in pepper and tobacco there was significant difference between the two races at 1-2 and 3-4 true leave stages (Fig. 3.4 a, b, c) but not at 5-6 true leave stage (Fig. 3.4 d,e).

At a given stage of inoculation and race when disease index between hosts is compared, no significant difference was observed between hosts at 1-2 and 3-4 true leave stages for race 1 (Fig. 3.4 f) . However, at 5-6 true leave stage, disease on pepper and tobacco is significantly lower than that of potato, tomato and eggplant. On the other hand, for race 3 at all stages of growth disease on pepper and tobacco was significantly lower than disease on potato, tomato and eggplant (Fig. 3.4 g). This was because the two hosts are resistant to race 3 strain. In strict sense, there was no disease developed on pepper and tobacco at all growth stages when inoculated by race 3 strain, while when inoculated with race 1 strain high amount of disease developed at 1-2 and 3-4 true leaf stage but no disease developed at 5-6 true leaf stage.

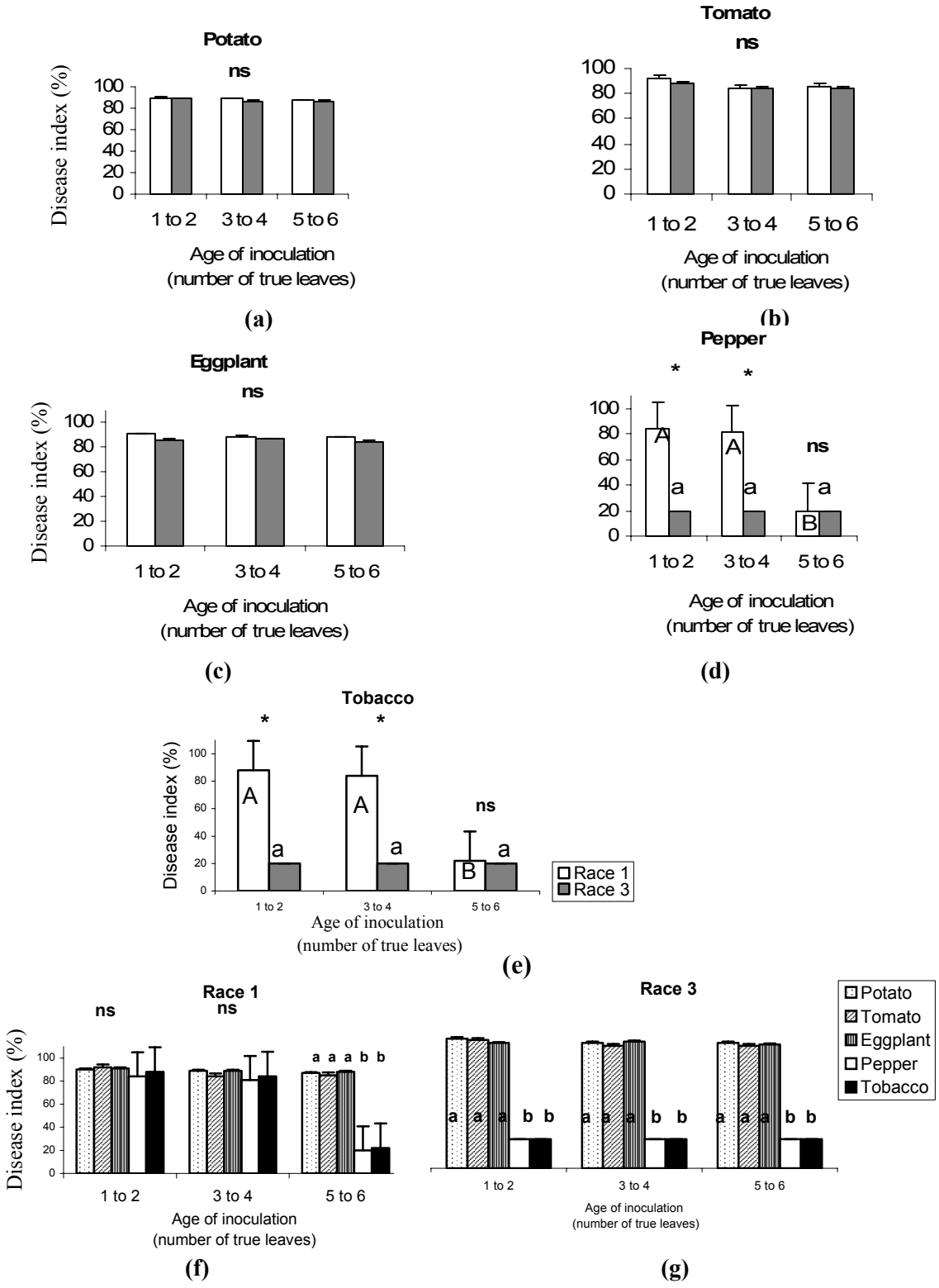


Fig. 3.4. Disease severity (% + SE) on five differential hosts caused by race 1 and race 3 strains of *Ralstonia solanacearum* when inoculated at different growth stages of the host plants. * in (d) and (e) shows presence of significant difference ($P < 0.002$) in disease index between race 1 and 3 at a given age of inoculation. Non significant differences are indicated by ns.

3.4. Discussion

Several authors have grouped *R. solanacearum* strains into different races on the basis of their pathogenicity on differential hosts (Buddenhagen *et al.,* 1962; Kelman and Person, 1961; He *et al.,* 1983; Horita and Tsuchiya, 2001). Lozano and Sequeira (1970) also described that differences in reactions of tobacco leaves to infiltration with isolates of *R. solanacearum* can be used as a simple and quick means to determine the race of any particular virulent strain of this pathogen. In our experiment pathogenicity test on differential hosts has been used in determining the races of Ethiopian *R. solanacearum* strains. Moreover, HR of tobacco leaves was compared with the results of pathogenicity test.

Generally, *R. solanacearum* strains are classified in race 1 if they attack tobacco, tomato, and many other solanaceous crops, and certain diploid bananas; race 2 if they infect triploid bananas and *Heliconia* spp; race 3 if potato and tomato and rarely other solanaceous plants are infected; and race 4 if mulberry is attacked (Buddenhagen *et al.,* 1962; He *et al.,* 1983; OEPP/EPPO, 2004). Accordingly, strains of *R. solanacearum* from Ethiopia were grouped in the present study in race 1 and race 3, the majority of the strains being in race 3 (Table 3.1). Race 1 strains were heterogeneous in pathogenicity and grouped into two pathogenic groups, while those in race 3 were found to be homogenous falling in the same pathogenicity group. Race 1 strains were from potato, tomato, and pepper and found to be pathogenic to all or four of the five solanaceaous crops tested (potato, tomato, eggplant, pepper and tobacco), while race 3 strains were from potato and tomato and were pathogenic to potato, tomato and eggplant.

This is the first report of race 1 strains of *R. solanacearum* in Ethiopia as only race 3 strains that belong to biovar II had been reported from strains collected from potato and tomato in

previous studies in Ethiopia (Yaynu, 1989). However, it is not surprising that race 1 representatives were among those collected in Ethiopia, because race 1 occurs in tropical areas all over the world and attacks tobacco, many other solanaceous crops and many hosts in other plant families (Buddenhagen *et al.,* 1962). Another supporting fact is that, Ethiopia has been introducing potato tubers from different parts of the world, particularly from International Potato Center (CIP) in Peru, for improving the productivity and resistance of the local germplasm (Berga *et al.,* 1994). Thus it may be possible that race 1 strains have been introduced to Ethiopia through latently infected potato tubers from other parts of the world. Epidemiological studies demonstrate that dissemination of the bacterium is mainly by latently infected tubers or by contaminated irrigation water (Williamson *et al.,* 2002).

On the basis of HR on tobacco leaf, Ethiopian *R. solanacearum* strains could be grouped into three distinct groups (Table 3.2). Group 1 produced slow spreading necrosis (N) on tobacco leaves and embraces most strains that failed in race 1; group 2 strains caused hypersensitivity reaction (HR) and it includes only four strains from race 1; and group 3 strains caused yellowish discoloration (Y) and embraces all of race 3 strains. Lozano and Sequeira (1970) reported HR only from strains belonging to race 2, while in our study although there were no strains that were grouped into race 2, four strains in race 1 which were non pathogenic to tobacco induced HR on tobacco leaf (Table 3.2). Our results are in agreement with that of Horita and Tsuchiya (2001) and He *et al.* (1983) who also found HR from strains that belonged to race 1 but were non pathogenic to tobacco.

Yaynu (1989) suggested from his findings in Ethiopia that fields which cannot be planted with potato and tomato because of *R. solanacearum* problems may be used for pepper or tobacco as these two plants were not infected by any of his isolates. Nevertheless, it is of considerable concern that race 1 strains identified in our study have a wide host range and

were pathogenic to pepper and tobacco. Thus this finding raises the disturbing possibility that the bacterium could inflict heavy damage on other crops like pepper, tobacco and other plant species in Ethiopia. Thus management practices such as cultural practices that address not only race 3 strains but also the new race strains should be searched in Ethiopia.

Strains of *R. solanacearum* are differentiated into races by inoculating the differential hosts mainly at 3 to 4 true leave stages (He *et al.,* 1983; Hayward 1991; Horita and Tsuchiya, 2001; OEPP/EPPO, 2004). In our study, we made investigations with the hypothesis that staggering time of inoculation may affect disease development on hosts and classifying of strains into races. Our result clearly showed that time of inoculation affects disease development on differential hosts depending on type of host and race. In potato, tomato and eggplant, disease was not significantly affected by age of inoculation and type of race. However, in pepper and tobacco, disease development by virulent strain (race 1) was significantly reduced when inoculation was made at 4 to 5 true leave stage as compared to at earlier stages. At this stage (although the two hosts are susceptible when inoculation was made at early stages of inoculation) the strain failed to express symptoms on pepper and tobacco except that only stunting of growth and deformation of leaves were slightly observed in few strains.

From this result, it can be concluded that age of inoculation has no effect on the expression of symptoms on potato, tomato and eggplant, while significantly affects expression of symptoms in tobacco and pepper. Therefore, in a study where the ultimate objective is to know pathogenicity of strains to classify them into races, delayed inoculation may lead to misleading result in pepper and tobacco as susceptible hosts may remain healthy as if they are non hosts and strains will be grouped into another race than their own (e.g., into race 3 while they are race 1 strains). Secondly, this result is an indication that pepper and tobacco plants can escape infection from *R. solanacearum* if mechanisms that delay contact between the host

and the pathogen could be designed. One such mechanism can be delayed transplanting of pepper where the seedlings can be raised on *R. solanacearum* free nursery soil and transplanted to *R. solanacearum* infected fields.

The fact that pepper and tobacco are susceptible to race 1 when inoculated at 1 to 2 and 3 to 4 true leave stages but resistant at 5 to 6 leave stages indicates that, resistance in the two hosts is related to age. The relationship between plant age and disease resistance has been investigated in many plant-pathogen systems (Koch and Mew, 1991; Chang *et al.,* 1992; Heath, 1993; Rupe and Gbur, 1995; Kus *et al.,* 2002). Some plants become more susceptible to certain pathogens as they develop (Miller, 1983); however, susceptibility decreases with increasing age in others (Koch and Mew, 1991; Roumen *et al.,* 1992; Kus *et al.,* 2002). Older plants display increased resistance in the wheat/*Puccinia recondite* f.sp. *tritici* (Pretorious *et al.,* 1988) and tobacco/*Pernospora tabacina* (Reuveni *et al.,* 1986) interactions. Kus *et al.* (2002) found out that Arabidopsis becomes more resistant to virulent *Pseudomonas syringae* (pv *tomato* or *maculicola*) as plants mature.

The actual mechanisms responsible for the different forms of age related resistance have been studied in a preliminary manner only in few cases (Kus *et al.,* 2002). Lazarovits *et al.* (1981) observed a positive correlation between increasing plant age, glyceollin production, and resistance to *Phytophthora megasperma* var *sojae* in soybean. A similar correlation was observed for the accumulation of capsidiol in mature pepper plants in response to *Phytophthora capsici* (Hwang, 1995). In some cases resistance in old plants results from the accumulation of toxic antimicrobial compounds during the life cycle of the plant. Studies demonstrate that a response in matured tobacco plants (Yalpani *et al.,* 1993) and Arabidopsis (Kus *et al.,* 2002) is associated with an increase in endogenous salicylic acid. Therefore, the resistance of pepper and tobacco in our study may also be due to such type of host reaction.

4 Genetic Characterization of Strains of *Ralstonia solanacearum* from Ethiopia by Repetitive Sequence-based Polymerase Chain Reaction (rep-PCR)

Abstract

The genetic diversity among Ethiopian strains of *Ralstonia solanacearum* (Smith) was assessed by repetitive sequence-based polymerase chain reaction (rep-PCR) method with BOX and ERIC primer sets. The study comprised 62 strains collected from potato, tomato and pepper from different localities of Ethiopia (43 were identified as biovar II race 3, and 19 as biovar I race 1) and five reference strains which were not Ethiopian strain. The rep-PCR defined two major groups (1 and 2) among Ethiopian strains at 55% similarity level each matching to a single biovar. Group 1 comprised biovar II and group 2 biovar I strains. At 90% similarity level, biovar II strains were grouped into five and biovar I into one. Third group was formed by two of the reference strains at 55% similarity level which were biovar III. Comparative analysis of rep-PCR confirmed the results of the previous studies which split the species into an "Americanum" division including biovar I and II strains and an "Asiaticum" division including biovar III strains. Based on cluster analysis, Ethiopian biovar I and II strains are assumed to be American origin. The study showed diversity in Ethiopian *R. solanacearum* population. This is valuable information for designing disease control strategies and for formulation of plant breeding programs.

4.1. Introduction

Ralstonia (Pseudomonas) solanacearum (Smith) (Yabucchi *et al.,* 1995) embraces a diverse array of populations that differ in host range, geographical distribution, pathogenicity, genetic characteristics and physiological properties. To describe this intraspecific variability, binary classification systems are used. Accordingly, the pathogen is divided into five races (Pegg and Moffet, 1971; He *et al.,* 1983) based on host range and five biovars (Hayward, 1964; He *et al.,* 1983; Hayward, 1994) based on utilization of three disaccharides and three hexose alcohols. There is considerable genetic variation among strains within each race or biovar (Cook *et al.,* 1989; Poussier *et al.,* 1999). The delineation of bacterial populations is a prerequisite to studying the epidemiology of pathogens and, ultimately, the development of control strategies.

More recently genomic finger printing protocols have been used in order to differentiate microorganisms. The DNA-based analysis such as restriction fragment length polymorphism (RFLP), 16S rDNA sequence repetitive sequence-based polymerase chain reaction (rep-PCR), pulsed-field gel electrophoresis (PFGE), etc. are effective in measuring the diversity and genetic relationship among *R. solanacearum* strains (Horita and Tsuchiya, 2001; Dookun *et al.,* 2001; Smith *et al.,* 1995; Poussier *et al.,* 1999; Fraser *et al.,* 2001; Horita *et al.,* 2005; Seal *et al.,* 1999; Lee *et al.,* 2001; Peters *et al.,* 2004).

In Ethiopia *R. solanacearum* is an important pathogen that affects particularly potato and tomato (Yaynu, 1989). Recently it has also been observed that this bacterium is attacking pepper plant in some areas of Ethiopia (personal observation). Characterization of the strains has been carried out on the basis of physiological and biochemical properties. However, no study has been done to investigate genetic variability in this pathogen. It was found to be

necessary to investigate genetic variability amongst the various strains using molecular techniques so as to have an understanding in the population structure of the bacterium in Ethiopia. The objective of this study was, therefore, to assess the genetic diversity of Ethiopian strains and predict their geographical origin based on repetitive fragment method.

The rep-PCR method uses primer sets referred to as enterobacterial repetitive intergenic consensus (ERIC) repetitive extragenic palindromic (REP) and BOX that yield genomic fingerprints specific to pathovars and strains of gram negative bacteria (Jaunet and Wang, 1999; Horita and Tsuchiya, 2001; Horita *et al.,* 2005; Poliakoff *et al.,* 2005). The BOX and ERIC primer sets generate a robust, reproducible and a highly discriminatory fingerprint (Rademaker and de Bruijn, Website Accessed in February 2006). In our study therefore BOX and ERIC primer sets were used for studying genetic variation in Ethiopian *R. solanacearum* strains.

4.2. Materials and Methods

4.2.1. Bacterial strains and growth conditions

The collection of 62 Ethiopian strains, which have been identified as *R. solanacearum* by TTC, tomato bioassay and species specific primers (Chapter 2) were used in this study. Moreover, five strains of *R. solanacearum* of the Göttingen Phytobacteriology Collection (GSPB) were included for comparison. Of the five reference strains, originally two were from Peru, two from Kenya and one from Cameroon. The strains used in this study have been listed in Table 4.1. All strains were maintained in long-term storage as suspensions in sterile distilled water at room temperature (Wullings *et al.,* 1998). For further use, they were revived

by plating on tetrazolium chloride (TTC) medium (Kelman, 1954) or on casamino acids peptone glucose (CPG) agar (Smith *et al.,* 1995) as mentioned in Chapter 2.

4.2.2. DNA extraction

Bacterial strains were grown on TTC agar and single colonies of the strain were transferred to CPG broth and grown overnight on rotary shaker at 150 rpm and 28°C. One ml of the culture (OD_{600} = 0.4) was used for extraction of genomic DNA. Bacterial DNA was extracted with a commercially available kit (Qiagen GmbH, Hilden, Germany) and used as a template for PCR.

4.2.3. PCR analysis

The primer sets: ERIC, ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'); and BOX, BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') used in this study have been described previously by Versalovic *et al.* (1991) and used by several other authors (e.g., Horita *et al.,* 2005; Sander *et al.,* 1998; Zhao *et al.,* 2000; Louws *et al.,* 1994; Peters *et al.,* 2004; Shutt, *et al.,* 2005). Primers were synthesized by Metabion International Ag, Martinsried, Germany. PCR amplification was performed in a final volume of 25 µl, consisting of 1 × PCR reaction buffer (Qiagen GmbH, Hilden, Germany), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 50 pmol of each primer, 2.5 U of DNA polymerase Takra *Taq* polymerase, and 1µl of the extracted DNA (about 25 ng template DNA). The PCR amplification was performed with an automated thermal cycler, Mastercycler®, (Eppendorf AG, Hamburg, Germany) programmed as follows: an initial denaturation step of 95°C for 2 min, followed by 30 cycles of 94°C for 30 s (ERIC) or 94°C for 1 min (BOX), 50°C for 1 min (ERIC) or 52°C for 1 min (BOX), and 65°C for 4

min with a final extension of 65°C for 5 min (ERIC) or 65°C for 2 min (BOX), followed by hold time at 4°C until samples were collected.

Amplified products (10 µl) were resolved by gel electrophoresis in 1.5% agarose gel in 1×TAE (10 mM Tris-HCl and 1mM EDTA) buffer, pH 8.0, at 120 voltage. The gel was stained with ethidium bromide (10 mg/ml) and the DNA fragments (bands) were detected by a UV trans-illumination and photographed under UV light using Polaroid 667 films. For confirmation of banding patterns, PCR experiments were repeated at least two times.

4.2.4. Data analysis

Rep-PCR finger print profiles were used to measure genetic similarity among strains. Each band with a different electrophoretic mobility was assigned a position number and scored as either 1 or 0 based on the presence or absence of the band, respectively, for this position. Variations in intensity of bands among isolates were not considered as differences. Similarity coefficients for all possible pairs of strains based on the finger print groups were estimated by Dice method (Dice, 1945). Dendrogram was generated from the similarity coefficient data by the unweighted pair group method with arithmetic averages (UPGMA) clustering (Sneath and Sokal, 1973).

4.3. Results

Rep-PCR genomic fingerprints of 62 Ethiopian strains and 5 reference strains were generated by BOX and ERIC primer sets (Fig 4.1a and b). Similarity coefficients were calculated based on the finger prints and a dendrogram was constructed (Fig. 4.2). Both BOX and ERIC primer sets gave clear genomic PCR profiles that were highly reproducible. BOX primer produced 4

to 10 bands per isolate within the range of 0.5 to 5 kb molecular size, whereas the REP primers generated 3 to 11 bands per isolate within a range of approximately 0.15 to 3.0 kb. Polymorphic bands were clearly observed by both primer sets helping differentiation into groups. Only a single monomorphic band was observed with ERIC primer sets at about 0.7 kb, while no monomorphism was detected with BOX primer. (A band was considered as polymorphic if any of the individual strains lacks it and as monomorphic if all of the strains have it).

After duplicate analysis, both BOX and ERIC-PCR defined three major groups (group 1, 2 and 3) at 55% similarity level with confidence (Fig. 4.2). The number of strains in groups 1, 2, and 3 was 45, 20 and 2, respectively (Table 4.1). Each group contained strains belonging to the same biovar, with the exception of group 2, which included strains of biovar I and III (only one strain from the latter). Group 1 contained all biovar II strains, group 2 all biovar I strains and one biovar III strain (the exception), and group 3 two strains from biovar III. Average similarities within groups 1, 2 and 3 were more than 57%, 94%, and 55%, respectively. On the contrary, average similarity between group 1 and 2 was 37%, 2 and 3, 31%, and 1 and 3, 31%. Race wise, all group 1 strains are race 3, while all group 2 and 3 strains are race 1.

At a similarity level of 90%, group 1 strains were distributed over five sub groups (1a, 1b,1c,1d and 1e) with majority (84%) of the strains in sub group 1a. Group 2 strains were grouped together and group 3 strains were grouped into two sub groups (3a and b) with one strain in each group. Generally, Ethiopian strains were put under group 1 (the majority) and group 2. In group 3 were only two reference strains; one from Kenya and the other from Peru.

At an average of 35% similarity level, the whole population could be grouped into two clusters. Cluster 1 consisted all biovar II and I strains (with exception of one strain), while cluster 2 contained biovar III strains.

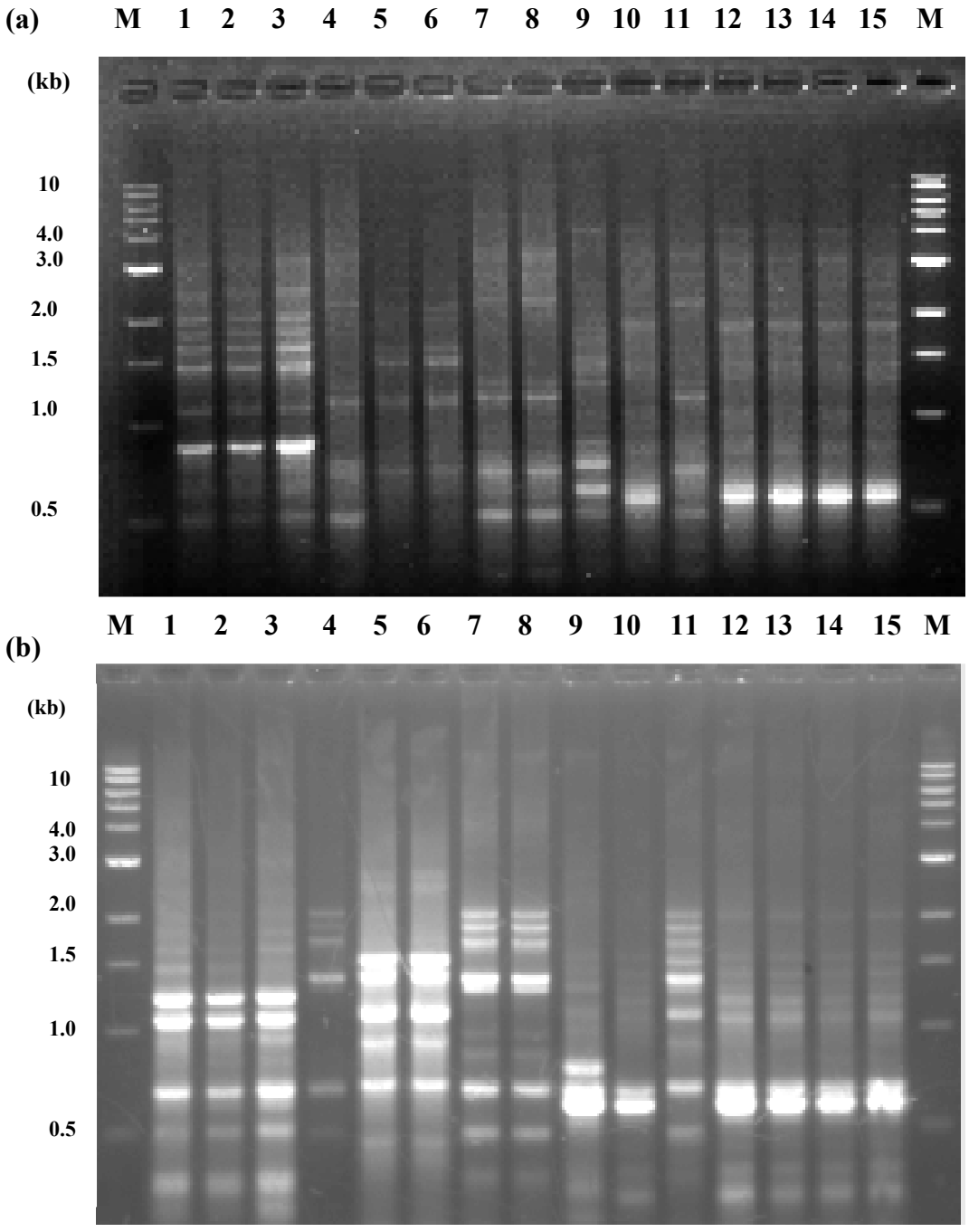


Fig. 4.1. Agarose gel bands showing representative patterns of Ethiopian *Ralstonia solanacearum* strains and five reference strains from Göttingen Collection of Phytopathogenic Bacteria (GSPB), Göttingen, Germany, generated by repetitive sequence-based polymerase chain reaction with (a) BOX and (b) ERIC primers. Lane M, DNA molecular size marker (kilo base DNA ladder); Sizes are indicated on the left in kilo base pairs (kb); lanes: 1, Pot 34; 2, Tom 58; 3, GSPB 2791; 4, GSPB 2695; 5, Pot 9JU; 6, Pot 1080; 7, Pot 29JU; 8, Pot 60; 9, GSPB 2690; 10, GSPB 2709; 11, Pot 17III; 12, GSPB 2792; 13, Pot 91; 14, Pot 53; 15, Pep 61.

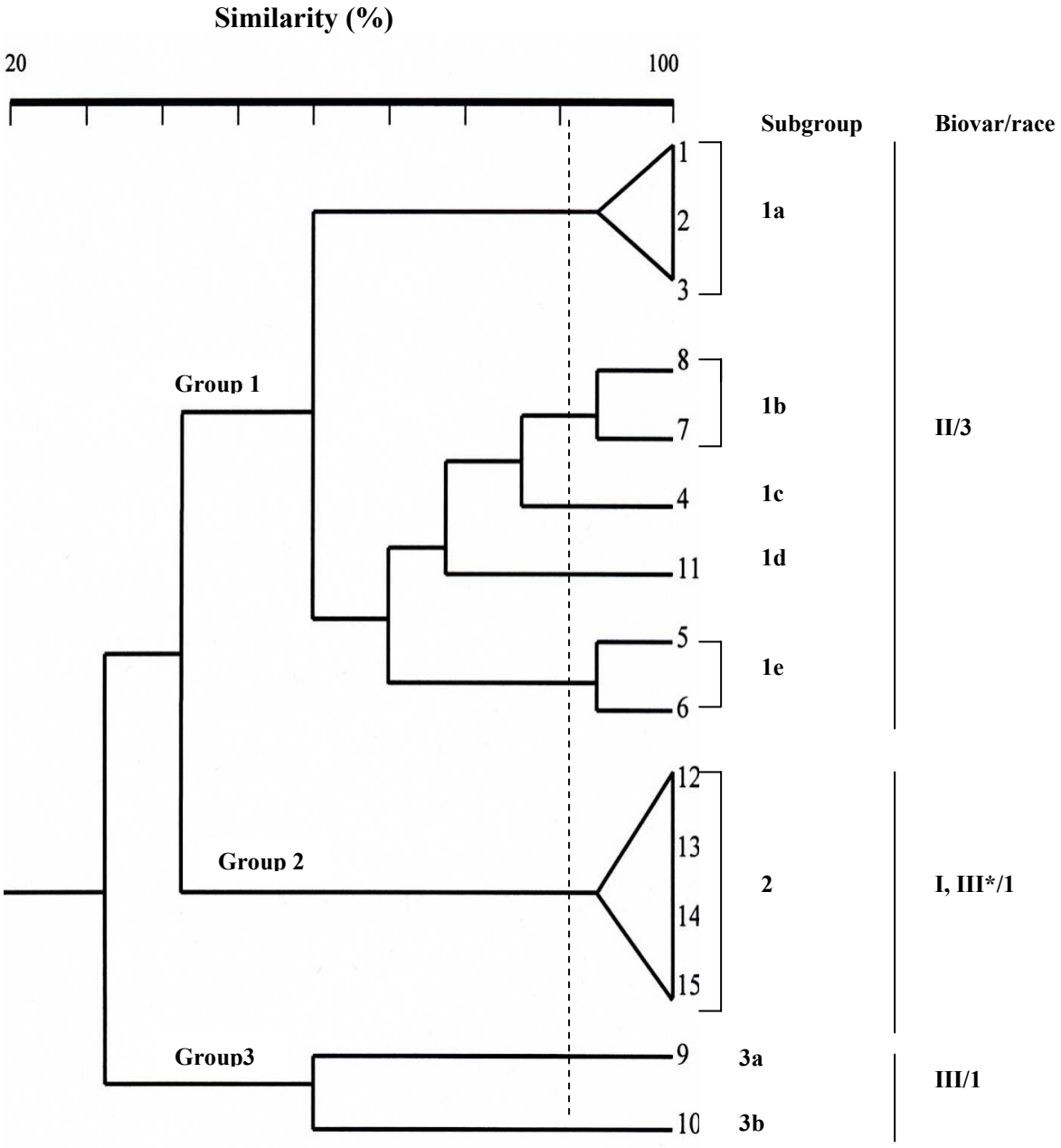


Fig. 4.2. Genetic diversity of Ethiopian *Ralstonia solanacearum* strains and five reference strains from Göttingen Phytobacteriology Collection (GSPB), Göttingen, Germany, on the basis of rep-PCR. A similarity coefficient was calculated based on fingerprints using the Dice (1945) coefficient. A dendrogram was constructed by unweighted pair group method with arithmetic average (UPGMA) clustering. Numbers at the right tips of dendrogram show lane numbers presented in Fig.4.2. Sub group, biovar and race are given on the right. The * shows that only one strain from biovar III has been put in the group.

Table 4.1. Strains of *Ralstonia solanacearum* used in the study

Strain name	Geographical origin	Original host	Pathogenic group	Race	Biovar	rep-PCR group
Pot 1	Guder, Ethiopia	Potato	3	3	II	1a
Pot 5	Guder, Ethiopia	"	3	3	II	
Pot 34	Gedo, Ethiopia	"	3	3	II	
Pot 5 II	Holeta, Ethiopia	"	3	3	II	
Pot 9II	Holeta, Ethiopia	"	3	3	II	
Pot 10II	Holeta, Ethiopia	"	3	3	II	
Pot 15II	Guder, Ethiopia	"	3	3	II	
Pot 4JU	Jimma, Ethiopia	"	3	3	II	
Pot 6 JU	Jimma, Ethiopia	"	3	3	II	
Pot 6III	Kejo, Ethiopia	"	3	3	II	
Pot 10III	Kejo, Ethiopia	"	3	3	II	
Pot 20III	Arjo, Ethiopia	"	3	3	II	
Pot 21 III	Arjo, Ethiopia	"	3	3	II	
Pot 40	Ginchi, Ethiopia	"	3	3	II	
Pot 56	Shashemene, Ethiopia	"	3	3	II	
Pot 57	Shashemene, Ethiopia	"	3	3	II	
Pot 61	Awassa, Ethiopia	"	3	3	II	
Pot 65	Jimma, Ethiopia	"	3	3	II	
Pot 66	Jimma Ethiopia	"	3	3	II	
Pot 68	Jimma, Ethiopia	"	3	3	II	
Pot 70	Jimma, Ethiopia	"	3	3	II	
Pot 71	Jimma, Ethiopia	"	3	3	II	
Pot 81	Kombolcha, Ethiopia	"	3	3	II	
Pot 84	Ambo, Ethiopia	"	3	3	II	
Pot 86	Kejo, Ethiopia	"	3	3	II	
Pot 92	Shashemene, Ethiopia	"	3	3	II	
Pot 93	Shashemene, Ethiopia	"	3	3	II	
Tom 3	Guder, Ethiopia	Tomato	3	3	II	
Tom 1II	Holeta, Ethiopia	"	3	3	II	
Tom 56	Adam Tulu, Ethiopia	"	3	3	II	
Tom 58	Guder, Ethiopia	"	3	3	II	
Tom 88	Ziway, Ethiopia	"	3	3	II	
Pot 1076PPRC*	Kombolcha, Ethiopia	Potato	3	3	II	
Pot 1079PPRC*	Qarsa, Ethiopia	"	3	3	II	
Pot 262APPRC*	Ambo, Ethiopia	"	3	3	II	
Pot 1091PPRC*	Agaro, Ethiopia	"	3	3	II	
Tom 768PPRC*	Ziway, Ethiopia	Tomato	3	3	II	
GSPB 2791**	Peru	Potato	3	3	II	
Pot 16III	Bako, Ethiopia	Potato	3	3	II	1b
Pot 29JU	Jimma, Ethiopia	"	3	3	II	
Pot 60	Shasemene, Ethiopia	"	3	3	II	
GSPB 2695**	Kenya	Tomato	3	3	II	1c
Pot 17III	Bako, Ethiopia	Potato	3	3	II	1d
Pot 9JU	Jimma, Ethiopia	"	3	3	II	1e
Pot 1080PPRC*	Qarsa, Ethiopia	"	3	3	II	

Continued on next page

Strain name	Geographical origin	Original host	Pathogenic group	Race	Biovar	rep-PCR group
Pot 2JU	Jimma, Ethiopia	Potato	1	1	I	2
Pot 4II	Holeta, Ethiopia	"	1	1	I	
Pot 8 JU	Jimma, Ethiopia	"	1	1	I	
Pot 31 JU	Jimma, Ethiopia	"	1	1	I	
Pot 46	Jeldu, Ethiopia	"	1	1	I	
Pot 48	Ginchi, Ethiopia	"	1	1	I	
Pot 50	Ginchi, Ethiopia	"	1	1	I	
Pot 55	Shashemene, Ethiopia	"	1	1	I	
Pot 58	Shashemene, Ethiopia	"	1	1	I	
Pot 59	Shashemene, Ethiopia	"	1	1	I	
Pot 94	Shashemene, Ethiopia	"	1	1	I	
Tom 6II	Holeta, Ethiopia	Tomato	1	1	I	
Tom 53	Shashemene, Ethiopia	"	1	1	I	
Pep 58	Guder, Ethiopia	Pepper	1	1	I	
Pep 61	Guder, Ethiopia	"	1	1	I	
Pep 7	Guder, Ethiopia	"	2	1	I	
Pot 42	Jeldu, Ethiopia	Potato	2	1	I	
Pot 62	Awassa, Ethiopia	"	2	1	I	
Pot 91	Shashemene, Ethiopia	"	2	1	I	
GSPB 2792**	Cameroon	"	1	1	III	
GSPB 2690**	Kenya	Pepper	1	1	III	3a
GSPB 2709**	Peru	Potato	1	1	III	3b

All the strains were collections of this study except those with * which were obtained from Plant Protection Research Center (PPRC), Ambo, Ethiopia, and those with ** which are procured from Göttingen Collection of Phytopathogenic Bacteria (GSPB),Göttingen, Germany

4.4. Discussion

Although there have been already few studies on biochemical and pathogenic properties of Ethiopian *R. solanacearum* strains (Yaynu, 1989), no study has been undertaken on their genetic characterization. The objective of this study was therefore to elucidate genetic variation among Ethiopian *R. soalanacearum* strains and define their population structure in comparison with previous results found in other parts of the world.

Rep-PCR analysis has been used to differentiate a great number of species and strains of phytopathogenic bacteria (Horita and Tsuchiya, 2001; Peters *et al.,* 2004; Trindade *et al.,* 2005; Horita *et al.,* 2005; Poliakoff *et al.,* 2005). Here we also used rep-PCR (BOX and ERIC) analysis to detect genetic diversity of Ethiopian strains along with five reference strains. Each primer set (BOX and ERIC) gave repeatable and distinct patterns (Fig. 4.1a and b) enabling differentiation of strains into groups. In the dendrogram based on the rep-PCR finger prints three main groups were found, each group representing a biovar except one strain (Fig. 4.2). Accordingly, all biovar II strains from Ethiopia were grouped into group 1, biovar I strains into group 2, and biovar III strains into group 3 (except one strain which was grouped to group 2 while it is biovar III).

In a study with strains from many parts of the world, Smith *et al.* (1995) and Poussier *et al.* (1999) have shown that biovar II is the most homogenous group and gather in one group by RFLP and rep-PCR. In our investigation also most of biovar II strains were pooled in one subgroup (1a) at 90% similarity level. However, some of the strains formed separate cluster in contrast to what has been mentioned by Smith *et al.* (1995) and Poussier *et al.* (1999). This disagreement to the previous findings may be due to the existence of genetic changes in strains with time or the world's population of *R. solanacearum* was not well represented in

those previous studies. The fact that most of the studies were made mainly on strains obtained from preserved collections of phytopathogenic bacteria may also reduce reflection of the actual population as they may be collected long time ago.

As opposite to biovar II, biovar I strains were reported to be heterogeneous population by several authors (e.g., Smith *et al.,* 1995; Poussier *et al.,* 1999; Horita and Tsuchiya, 2001). In the present study, however, biovar I strains were clustered together indicating homogeneity of the population. In Chapter 2, it was indicated that biovar I strains are a new type for Ethiopia. Thus the lack of diversity in Ethiopian biovar I strains may be due to close genetic basis of biovar I strains introduced to Ethiopia. Nevertheless, as biovar I was represented only by 19 strains from Ethiopia in this study, it would be crucial to consider large number of strains from this group to substantiate this result.

Horita and Tsuchiya (2001) indicated that biovar III strains have low average similarity and fall into five groups. Moreover, Poussier *et al.* (1999) has shown that biovar III strains cluster into two groups. Similarly, our cluster analysis grouped biovar III strains into three sub groups (3a, 3b, and 2) at 95% similarity level indicating their diversity.

Cook *et al.* (1989) assessed genetic diversity of *R. solanacearum* strains collected from Africa, America, Asia, Australia and Europe by RFLP method and revealed two main divisions. Division I comprised all members of biovars III, IV, and V, and is suggested to be of Asian origin (Asiaticum). Division II contained all members of biovars I and II, and is proposed to be of American origin (Americanum). Similarly, Taghavi *et al.* (1996) analyzed 16S rRNA gene sequences of *R. solanacearum* and related bacterial species and identified two divisions that correspond to results obtained from RFLP analysis by Cook *et al.* (1989). In another study made by Poussier *et al.* (1999) with African and worldwide strains of *R.*

solanacearum by RFLP, the dendrogram confirmed separation of *R. solanacearum* into two. However, the distribution of biovar I strains did not agree completely with the scheme proposed by Cook *et al.* (1989). In his result African biovar 1 strains were related to biovar III and IV strains and grouped to the Asiaticum division, while American biovar strains were related to biovar II strains and belonged to the Americanum division.

Our rep-PCR results closely correspond with the divisions proposed by Cook *et al.* (1989), as our group 1 and 2 could be clustered together at 25% similarity level, while group 3 formed another cluster. Thus biovar I and II strains could be classified in the Americanum division, while biovar III strains in Asiaticum division except that the Cameroonian strain (GSPB 2792) from biovar III was grouped into Americanum division. This further implies that Ethiopian biovar I which we reported as new introduction to Ethiopia (Chapter 2) and the previously existing biovar II strains are American origin. Berga *et al.* (1994) indicated that Ethiopia has introduced several thousands of potato genotypes mainly from International Potato Centre (CIP), Peru, in South America to improve potato productivity in the country. Thus introduction of the disease to Ethiopia may be through latently infected potato tubers.

R. solanacearum can be widely disseminated on vegetative propagating material (tomato, *Heliconia*, banana, and potato), and bacterial wilt of potato is spread locally and internationally by latently infected potato tubers (Hayward, 1991). Buddenhagen (1985) suggested that race 3 (biovar II) originated in the Andean region of South America where potato also originated and that the occurrence of race 3 outside South America was the result of human distribution of infected potato tubers. Several reports (Gillings and Fahy, 1993; Smith *et al.,* 1995; Poussier *et al.,* 1999; Horita and Tsuchiya, 2001) using DNA-based techniques (RFLP, rep-PCR, PFGE) support this hypothesis.

The only strain that violated the assumption of Cook *et al.* (1989) was a Cameroonian isolate GSPB 2792 which was put under division II (Americanum) while it was expected in division I. The bio-data of the strain from Göttingen Collection showed that it belongs to biovar III and our study in chapter 2 confirmed the same. Thus the relatedness of the strain to biovar I (in group 2) than biovar III (in group 3) strains (Fig. 4.2) may indicate that it has co-evolved from biovar I strains and may be American origin.

In general, the data presented here show diversity in Ethiopian *R. solanacearum* population as they cluster into two groups (1 and 2) at 55% and into six (1a to1e and 2) at 90% similarity level (Fig. 4.2). This suggests that control strategies may not be equally effective for all the grouped bacterial strains. Thus the information can be considered as valuable for designing control strategies for Ethiopian conditions. It could also help plant breeders for designing plant breeding programs.

5 Studies on Biocontrol of *Ralstonia solanacearum* with Bacterial Antagonists

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 rhizospheric bacteria, most of them collected from Ethiopia, were screened against Ethiopian *R. solanacearum* strains. 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* test in greenhouse. In greenhouse, soil and tomato seedlings were treated with the antagonists and their effects studied. The study showed that APF1 and B2G strains significantly reduce disease incidence and severity and increase weight of tomato plant. APF2 strain also significantly reduces the disease and increases plant weight but not as consistently effective as APF1 and B2G strains. Incidence area under progress curve (AUDPC) was reduced by 60, 56, and 50% in APF1, B2G and APF2 strains, respectively, while severity AUDPC was reduced by 65, 54, and 55%, respectively. Plant dry weight increases in APF1, B2G and APF2 strains were 96, 75, and 57%, 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 mode of action in APF1 strain was found to be partly due to siderophore production. The study revealed that APF1 and B2G strains are promising strains whose effectiveness should be further checked in field conditions and mode of action investigated in detail.

5.1. Introduction

Ralstonia solanacearum (Smith) (Yabucchi *et al.,* 1995) is an important soilborne bacterial plant pathogen with a worldwide distribution and a large host range of more than 200 species in 50 families (Hayward, 1995). Some of its economically important plant hosts include tomato, potato, eggplant, pepper, tobacco, banana, chilli, and peanut (French and Sequeira, 1970; Hayward, 1995). In Ethiopia *R. solanacearum* is an important disease of potato and tomato (Yaynu, 1989) and its importance is increasing from time to time (Berga *et al.,* 2000).

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 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; Hartman and Elphinstone, 1994) often restricts the expression of resistance to specific regions (Thurstone, 1976). Hence, alternative control measures for the management of wilt disease caused by *R. solanacearum* need to be developed.

Biological control is an effective, safe, and environmentally friendly approach for plant disease management. 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 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; Wydra and Semrau, 2005), *Pseudomonas* spp. (Guo *et al.,* 2001; Priou *et al.,* 2005; Wydra and Semrau, 2005), 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. Moreover, as a result of heterogeneity within the species *R. solanacearum* (Elphinstone, 1992), no one biological control agent or methodology is likely to be universally effective. Thus in order to select effective biocontrol agents for Ethiopian strains, it is important to screen bacterial antagonists against these specific strains.

Several mechanisms have been proposed for suppression of pathogens by *Pseudomonas* and *Bacillus* spp. (Hessenmüller and Zeller, 1996; Blanco *et al.,* 2004; Ran *et al.,* 2005; Dwivedi and Johri, 2003). Pseudomonads provide a protective effect on the roots through antagonism towards phytopathogenic bacteria by producing metabolites such as: biosurfactants e.g., rhamnolipids (Stanghellini and Miller, 1997), hydrogen cyanide (Schippers *et al.,* 1991; Voisard *et al.,* 1989); lytic enzymes e.g., ß-1,3 glucanase, protease (Friedlender *et al.,* 1993; Berg, 1996); plant hormones and other plant growth promoting substances e.g., auxins, indole-3-acetic acid (Loper and Schroth, 1986), gibberellins (Ramamoorthy and Samiyappan, 2001) and 1-aminocyclopropane-1-carboxylate deaminase (Jacobson *et al.,* 1994); siderophores e.g., pyoverdins, pseudobactin and pyochelin (Leong, 1986; Duijff *et al.,* 1994; Dwivedi and Johri, 2003); antibiotics e.g., phenazines (Défago and Haas, 1990; Thomashow *et al.,* 1990), pyoluteorin (Kraus and Loper, 1995; Dwivedi and Johri, 2003), pyrrolnitrin

(Défago and Haas, 1990; Lambert *et al.,* 1987), and 2,4-Diacetyl phloroglucinol (Raaijmakers and Weller, 1998; Harrison *et al.,* 1993; Ran *et al.,* 2005).

Members of the genus *Bacillus* are also known to produce a wide range of secondary metabolites such as antibiotics, non-volatile and volatile compounds (Parke and Gurian-Sherman, 2001, Fiddaman and Rossall, 1993) and lytic enzymes (Frändberg and Schnürer, 1994). Some of the antibiotics produced by *Bacillus* spp. include: bacillomycin, mycobacillin, iturin A, surfactin, mycosubtilin, fungistatin, subsporin, bacilysin, chlorotetetain (Krebs *et al.,* 1998; Loeffler *et al.,* 1990; Asaka and Shoda, 1996; Yu *et al.,* 2002). *Streptomyces* spp. also produce antibiotic compounds and have protected a range of plant species from phytopathogenic organisms (Coombs *et al.,* 2004). Competition for space (Elad and Chet, 1987) and induction of systemic resistance (Pieterse *et al.,* 2001) are also involved in disease suppression by bacterial antagonists. Understanding of the mechanisms of microbial antagonists may gradually lead to effective utilization of beneficial microorganisms for control of plant diseases.

Therefore, the objectives of this study were to screen and select effective bacterial antagonists for the biological control of Ethiopian *R. solanacearum* and to make preliminary investigations on the mode of action of the antagonists.

5.2. Materials and Methods

5.2.1. 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 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.

5.2.2. Bacterial strain and culture conditions

As the majority of Ethiopian *R. solanacearum* strains were identified as biovar II race 3 strains (Chapters 2 and 3), a representative strain Pot 4JU out of this group 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 PCR as described in Chapter 2. Pot 4JU belongs to biovar II, as determined by testing for the ability to utilize three hexose alcohols (mannitol, sorbitol, and dulcitol) and three hexose alcohols (lactose, maltose, and cellobiose) (Hayward, 1964). The race was specified by inoculating 4-weeks old tomato, potato, eggplant, pepper and tobacco plants at leaf axle (Chapter 3), according to the standard set by Buddenhagen *et al.* (1962). Pot 4JU is pathogenic to potato, tomato and eggplant but not to pepper and tobacco. The strain was routinely cultured on CPG and TTC medium at 28°C for 48 h and temporarily stored in sterile water at room condition.

5.2.3. *In vitro* screening

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

Chloroform vapour method: Candidate antagonistic bacteria were spotted on KB and LB media at the outer margin of plates at four corners 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. Test pathogen was cultured in CPG broth on rotary shaker for 24 h and centrifuged at 10,000 rpm and cell pellets were diluted in 85% 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. Each combination of microorganisms, controls and culture media were replicated three times in a randomised complete block design and the experiment was repeated twice.

Agar-diffusion test: One-hundred µl 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 antagonists ($\approx 10^9$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.

5.2.4. Identification of the selected bacteria

Six bacterial strains with the highest inhibitory effect *in vitro* were selected and used during the study. Two of them were *Streptomyces setonii* RP87 and *Bacillus subtilis* B2G that were obtained from Rostock University, Germany, and included in our study. The remaining four were strains out of 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) by pigmentation on KB medium, Gram stain, oxidase and catalase test, starch hydrolysis, gelatine liquefaction and growth on carbon sources. The biochemical tests were carried out in the same way performed in Chapter 2.

5.2.5. Studies on mode of action

Preliminary studies on the mode of action of the selected antagonistic strains were carried out by detecting siderophore and extracellular protein production as follows:

Production of siderophore: Siderophore production was assessed by the $FeCl_3$ test (Nieland, 1981) by spotting the antagonists at four corners of iron deficient KB agar plates and of KB agar plates supplemented with 100 µM $FeCl_3$, respectively. After 24 h of incubation, the agar plates were inverted on chloroform vapour for 3 min and the antagonists destroyed. Then the plates were flooded with test strain (*R. solanacearum*) at 10^8 cfu/ml, dried by reabsorbing and incubated at 28°C. After 2-3 days of incubation, inhibition zone of *R. solanacearum* was measured and the difference between the iron deficient and iron rich plates was compared statistically. Increased inhibition zone in the iron deficient plate is an indication for siderophore production.

Production of extracelluar proteins: For the analysis of extracellular proteins, antagonistic strains were cultured in mannitol-glutamate (MG) broth (Keane *et al.,* 1970), a minimal medium containing the following nutrients (l^{-1}): 10 g mannitol, 2 g L-glutamic acid (monosodium salt), 0.5 g KH_2PO_4, 0.2 g NaCl and 0.2 g $MgSO_4.7H_2O$. The pH of MG medium was adjusted to 7.0 with 3M NaOH prior to autoclaving. Growth was performed at 18 and 28°C on rotary shaker at 150 rpm until the growth reaches stationary phase. Cultures were centrifuged at 15 000 rpm for 20 min and the supernatants collected and filtered through 0.2µm pore filter sterilizer to get cell free supernatant for determination of extracellular proteins.

Extracellular proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using a Mini-Protein Kit and visualized on 10% SDS polyacrylamide gel (20 µl on each lane) after staining with 0.05% Coomassie brilliant blue R-250. The same samples were run on four native gels to allow proper folding of the extracellular proteins and for determination of activities. The four gels were then: a) submersed in a 10% sucrose solution to test for levansucrase activity, b) blotted on a gel matrix which contained skim milk to test for protease activity, c) blotted on a gel matrix containing a lipid mixture to test for lipase activity, and d) blotted on a gel matrix containing cellulose to test for cellulase activity. As a control purified enzymes levansucrase, protease A, lipase and cellulase, respectively, were used at a concentration of 500 ng per lane.

5.2.6. Greenhouse studies

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 cv 'Matina' seeds were obtained from the HILD Samen GmbH, Marbach, Germany, and 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 greenhouse at temperatures of 24 to 28°C and 75 to 90% relative humidity and seedlings were watered with sterile water when necessary.

Bioassay: Six strains of bacteria with highest inhibition in *in vitro* test were further tested in greenhouse on tomato plants to evaluate their ability to control bacterial wilt *in vivo*. 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 J2-21M/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 gram of the sterilized potting medium was mixed with 75 ml of *R. solanacearum* Pot 4JU at 10^8 cfu/ml (≈1.2 x 10^6 cfu/g soil, dry weight) and filled 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 to 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 greenhouse at 24 to 28°C and 75 to 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.

Population dynamics of *R. solanacearum* in soil treated with antagonists: To determine 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 (Englebrecht, 1994). The medium constitutes: 10 g bacto peptone (Difco), 5ml 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% tetrazolim 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. Each sample was replicated four times. Since populations of bacteria approximate a log normal distribution (Loper *et al.,* 1984), values were log transformed before analysis to normalize variance.

Disease assessment: The percentage of diseased plants and disease severity were scored separately per pot, each containing two plants, at different time points based on wilting symptoms. Disease severity was assessed with a scale of 0 to 4 as described by Swanson *et al.* (2005), where 0 = no wilt, 1 = 1 to 25% leaf area wilted, 2 = 26 to 50% wilted, 3 = 51 to 75% wilted and 4 = 76 to 100% wilted or dead. For statistical analysis rated disease severity grades were converted to percentage severity index (PSI) with the formula described by Fininsa (2003) as :

$$PSI = S_{nr} \times 100/N_{pr} \times M_{sc},$$

where S_{nr} is the sum of numerical ratings, N_{pr} is the number of plants rated and M_{sc} is the maximum score on the scale. Moreover, the area under disease progress curve (AUDPC) was

calculated from percentage of disease incidence and severity according to the mid point rule (Garrett and Mundt, 2000) as:

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

where x_i is the percentage of disease severity or 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 severity or 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:

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

where D_C is disease of control and D_T is disease of the treatment group.

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 oven and weights evaluated for each treatment. Dry and fresh weights were used for data analysis.

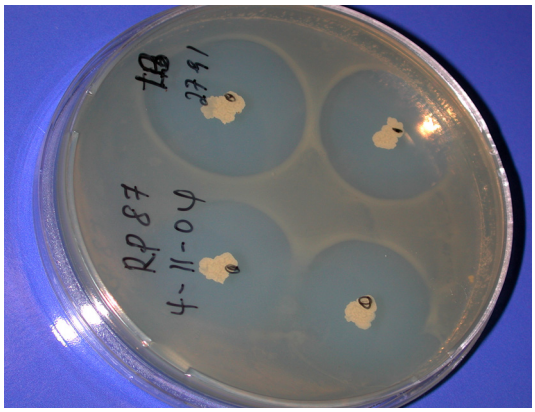
5.2.7. Statistical analysis

Data of experiments repeated over time were checked for homogeneity of variance using the HOVTEST = LEVENE option of ANOVA procedure in SAS version 8 (SAS Institute, 1999) and pooled only when variance homogeneity can be assumed. Single and interaction effects of factors were determined using the PROC GLM procedure of SAS. Correlation analysis was carried out using the PROC CORR procedure. Whenever significant interactions were observed between factors, the level of one factor was compared at each level of the other factor. When significant factor effects were detected by ANOVA, means at different levels of the respective factor were compared using the Tukey's mean comparison procedure. A significant level of $\alpha = 0.05$ was used in all analysis.

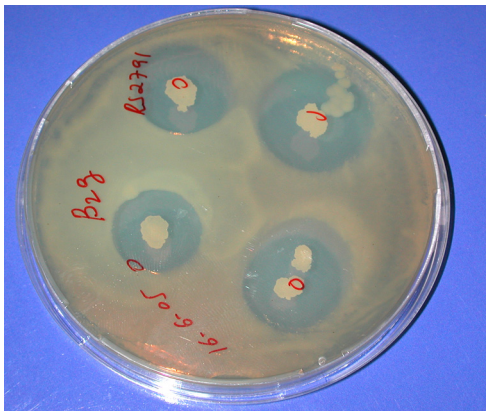
5.3. Results

5.3.1. *In vitro* inhibition

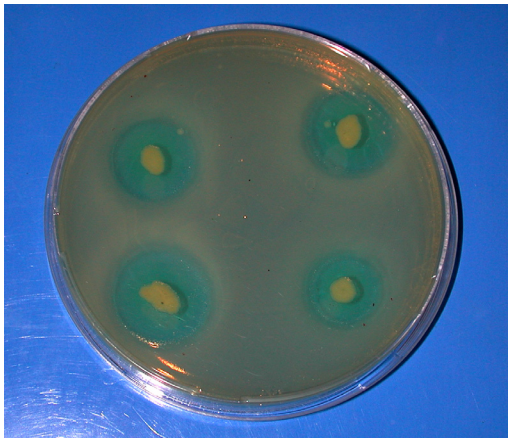
A total of 118 rhizospheric bacteria were screened against *R. solanacearum* strain Pot 4JU and 23 strains had inhibitory effect that ranged from an average of 0.5 to 11 mm radius of inhibition zone. Among those strains that had *in vitro* inhibitory effect, 16 (70%) of the strains were fluorescent under UV light when cultured on KB medium. Six strains with inhibition zone of 5 to 11 mm radius were selected and used for further study (Fig. 5.1). Two of the strains were *Bacillus subtilis* B2G and *Streptomyces setoni* RP87 which were from Rostock University, Germany, and four were out of the collection from Ethiopia and could be all identified as fluorescent pseudomonads based on the Bergey's manual. They were designated as APF1, APF2, APF3 and APF4.



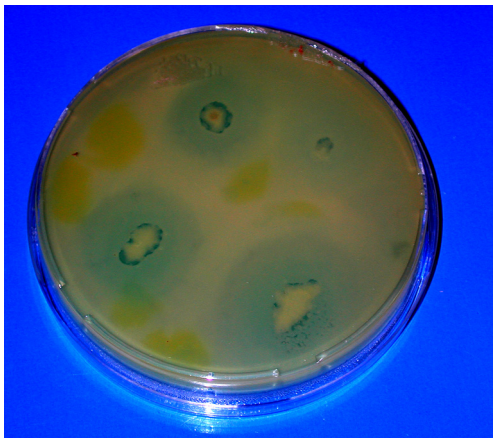
***Streptomyces setoni* RP87**



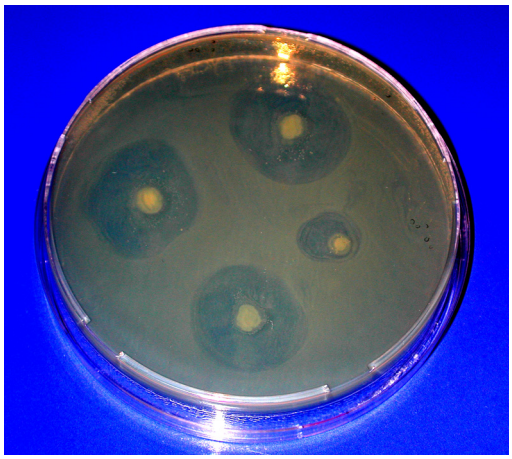
***Bacillus subtilis* B2G**



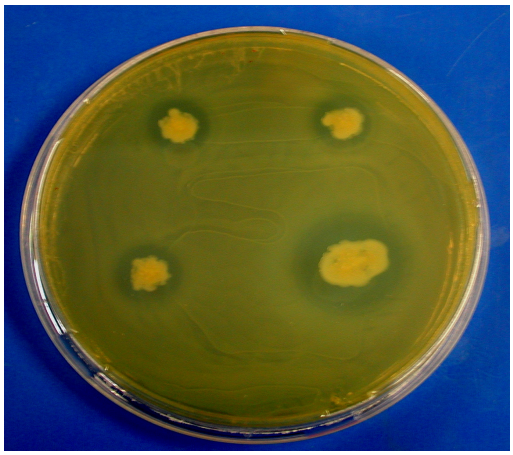
Fluorescent pseudomonad APF1



Fluorescent pseudomonad APF2



Fluorescent pseudomonad APF3



Fluorescent pseudomonad APF4

Fig. 5.1. *In vitro* inhibitory effects of six selected bacterial antagonists against *Ralstonia solanacearum* strain Pot 4JU on KB medium with chloroform vapour method. Antagonists were grown at four corners of the plate and killed by chloroform vapour. Then the plate was flooded with strain Pot 4JU and incubated for two or more days.

The results of *in vitro* tests indicated significant differences among antagonistic strains and between media (LB and KB) used. Methods of *in vitro* test (chloroform vapour and agar diffusion method) did not significantly affect growth inhibition (Table 5.1). The two-way interaction, medium by antagonist, was significant (df = 5, $F = 23.68$, $P < 0.0001$), indicating that the strains differed in inhibiting the pathogen depending on type of medium.

Table 5.1. ANOVA table for single and interaction effects of antagonistic bacteria in inhibiting *in vitro* growth of *Ralstonia solanacearum* strain Pot 4JU in different media and methods of tests

Source of variations	Df	*F*	*P*
Methods[1]	1	0.04	0.8423
Media[2]	1	125.44	<0.01
Antagonist	5	48.98	<0.0001
Method*medium	1	1.44	0.2360
Method*antagonist	5	1.62	0.1717
Medium*antagonist	5	23.68	<0.0001
Method*medium*antagonist	5	0.38	0.8573
Error	48	-	-

[1] Chloroform vapour and agar diffusion

[2] LB and KB

On LB medium, strains RP87 and B2G exhibited significantly higher inhibition zone than the other four strains, while on KB significantly higher inhibition was recorded by RP87, B2G, APF1 and APF2 (Table 5.2). Moreover, APF1 and APF2 exhibited significantly higher inhibition on KB than on LB. Inhibitory effects of strains RP87, B2G, APF3 and APF4 were not significantly affected by type of media.

Table 5.2. *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 LB and KB media

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, $\alpha = 0.05$)

5.3.2. Identification of strains

For antagonism study six strains were used. Two of them were identified species *Streptomyces setonii* RP87 and *Bacillus 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 fluorescent under UV light, oxidase, catalase, glucose, fructose, sucrose, and galactose positive (Table 5.3). 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 rhizosphere. Except RP87 and B2G, which were procured from Rostock University, Germany, others were collected from Ethiopia.

Table 5.3. Biochemical characteristics of the fluorescent pseudomonad antagonistic strains collected from Ethiopia

Biochemical tests	Antagonistic strains			
	APF1	APF2	APF3	APF4
KOH solubility	+	+	+	+
Fluorescence on KB	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
Gelatine liquefication	+	+	±	+
Starch hydrolysis	-	-	-	-
Carbon utilization:				
Fructose	+	+	+	+
Sucrose	+	+	+	+
Glucose	+	+	+	+
Galactose	+	+	+	±

+ = positive reaction; - = negative reaction; ± = variable reaction

5.3.3. Studies on the mode of action

Several mechanisms have been proposed for suppression of pathogens by antagonistic organisms among which siderophore and extracellular protein production are some. In our study siderophore and extracellular protein production were assessed to know if the antagonists were suppressing *R. solanacearum* wilt development through these mechanisms.

Siderophore production assessment: Table 5.4 shows the inhibitory activities of the antagonistic strains on KB medium in the presence and absence of 100 µM $FeCl_3$. The

inhibitory activities of pseudomonad APF1 (Fig. 5.2) and APF2 were significantly (df = 1, F = 57.13, 46.35, $P < 0.0001$) reduced (nearly by 50%) in iron supplemented KB plates as compared to KB plates deficient in iron. In all other strains, no significant difference in growth inhibition between iron supplemented and deficient KB media was found. This result indicated that pseudomonad APF1 and APF2 strains produce extracellular siderophore, which was strongly inhibitory to growth of *R. solanacearum* in iron-deficient condition.

Table 5.4. *In vitro* inhibition of growth of *Ralstonia solanacearum* by antagonistic strains (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and Fluorescent pseudomonads APF1, APF2, APF3, and APF4,) on KB medium in the absence or presence of 100 µM $FeCl_3$

Strain	Fe^-	Fe^+
RP87	10.34	10.45
B2G	10. 13	9.87
APF1	10.70	5.50*
APF2	9.90	4.80*
APF3	5.56	4.50
APF4	5.30	4.42

* indicates significant difference between inhibition in Fe^- and Fe^+ KB medium according to Tukey's test ($\alpha = 0.05$)

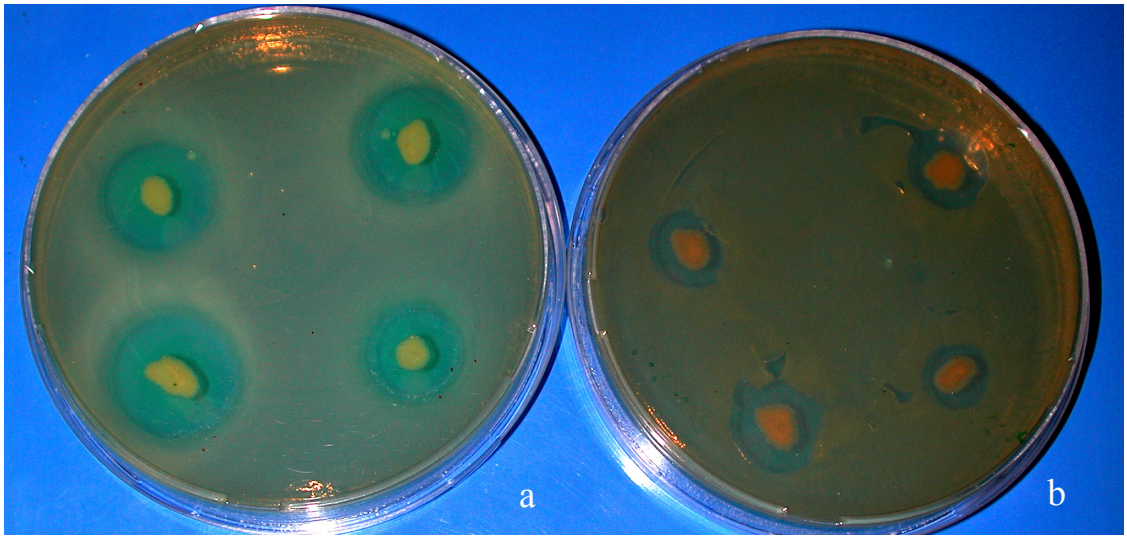


Fig. 5.2. *In vitro* inhibition of growth of *Ralstonia solanacearum* by antagonistic strain fluorescent pseudomonad APF1 on KB medium in the absence (a) and presence (b) of iron

Extracellular protein production: In Fig. 5.3, SDS-PAGE separation showed the production of extracellular proteins by all the fluorescent pseudomonad strains (APF1, APF2, APF3 and APF4) as shown in lanes 3 to 6. In all these strains bands of extracellular proteins were observed although the production was affected by temperature level in APF3 and APF4. In *S. setonii* RP87 and *B. subtilis* B2G no band was observed. After SDS-PAGE, further study was made to know if there are some lytic enzymes that play role in antagonism. This was by loading samples on native gels to determine activities for levansucrase, lipase, cellulase and protease which are some of lytic enzymes. The gel matrices of test samples remained empty (data not shown) suggesting that none of the strains produced any of the mentioned extracellular lytic enzymes.

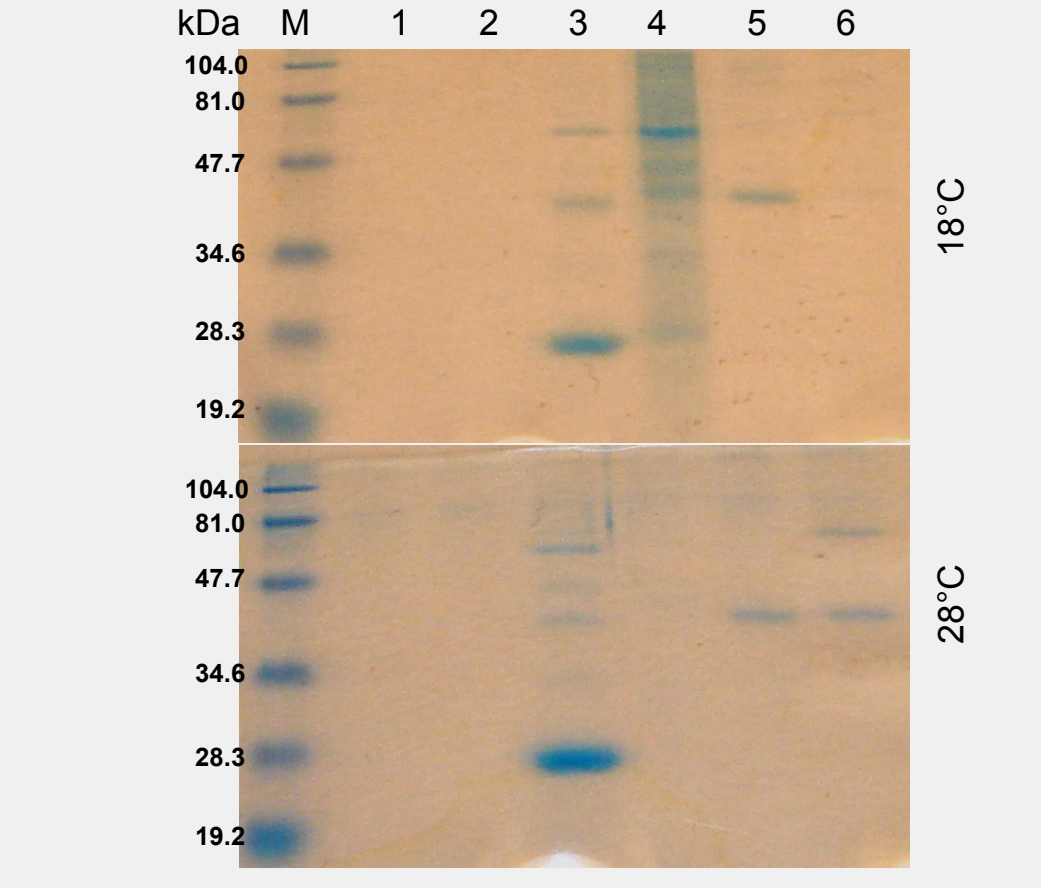


Fig. 5.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separation of extracellular proteins from cultures of bacterial antagonists. Lanes: 1, *Streptomyces setonii* RP87; 2, *Bacillus subtilis* B2G; 3-6, Fluorescent pseudomonads APF1, APF3, APF2, and APF4, respectively. Molecular mass of standard marker in kilo Daltons is shown on the left side. Temperatures of growth (18 and 28°C) are given on the right side.

5.3.4 Greenhouse studies

In greenhouse the effect of the selected antagonistic bacteria on wilt disease development and tomato plant growth and weight were assessed. Moreover, the effect of the antagonists on the population of *R. solanacearum* in soil was also tested.

Population dynamics of *R. solanacearum* in soil treated with antagonists: Population density of *R. solanacearum* (log cfu/g, dry weight) in the soil after incorporation of antagonists into potting medium is shown in Fig. 5.4. Starting from 0 up to 3 weeks after incorporation of antagonists into soil, population density of *R. solanacearum* declined in all treatments including the control. Later on, this tendency changed and population of the pathogen started to increase but differently among the treatments. Six weeks after soil treatment, population of the pathogen with pseudomonad APF1 and *B. subtilis* B2G was significantly (df = 6, $F = 5.29$, $P = 0.0018$) lower than the control. However, 9 weeks after soil treatment population of the pathogen is significantly (df = 6, $F = 7.25$, $P = 0.0002$) lower only in soil treated by pseudomonad APF1 strain. Generally, population density of *R. solanacearum* in soil was reduced most in soil that was treated with pseudomonad APF1 and *B. subtilis* B2G followed by pseudomonad APF2 strain. Per cent reductions of *R. solanacearum* population with pseudomonad APF1 and *B. subtilis* B2G strains were 44 and 45%, respectively, at 6 weeks after treatment and 35 and 26%, respectively, at 9 weeks.

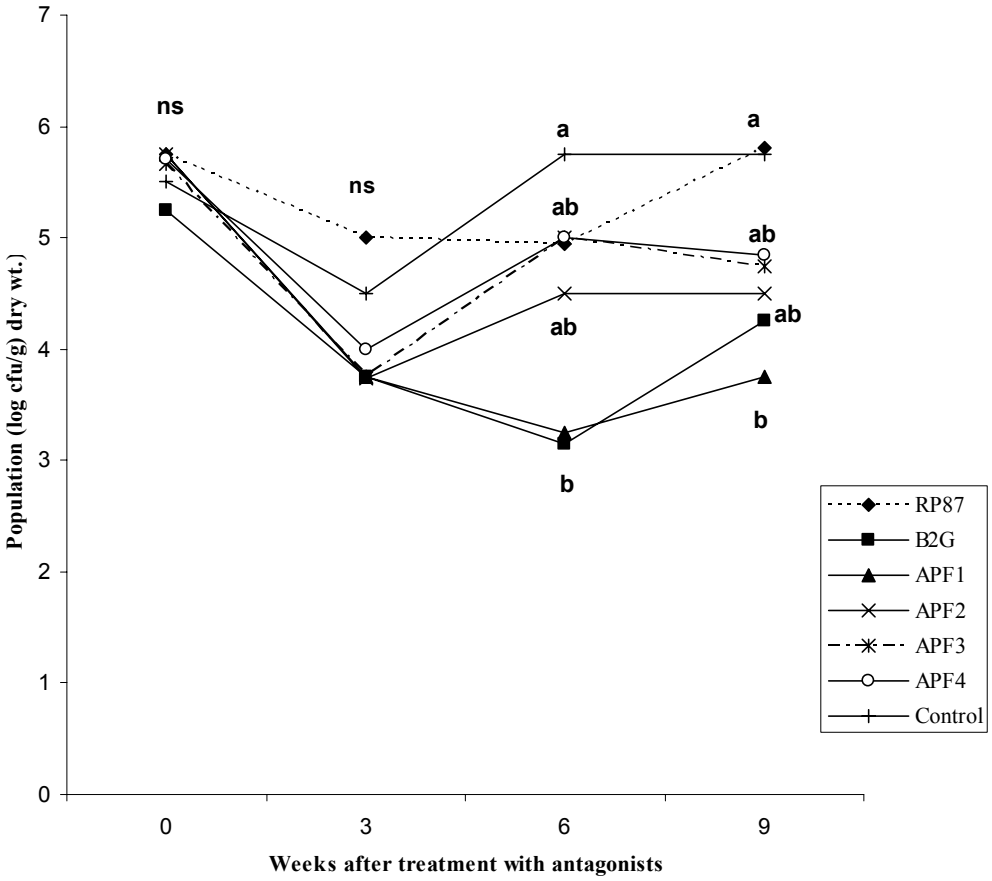


Fig. 5.4. Changes in population density of *Ralstonia solanacearum* in the rhizosphere of tomato at different intervals after introduction of bacterial antagonists (*Streptomyces setonii* RP87, *Bacillus subtilis*B2G, and fluorescent pseudomonads APF1, APF2, APF3 and APF4) into the potting medium. Potting 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.

Disease development: The *in vivo* efficacy of selected antagonists for the control of *R. solanacearum* wilt in tomato plants was evaluated under greenhouse conditions. Progress of wilt during greenhouse experiment is shown in Fig. 5.5. Initially wilt symptoms appeared 21 days after transplanting of the seedlings in treated soil. Incidence and severity progresses were closely similar and there was no significant difference among treatments until 33 days after transplanting. However, at 45 days after transplanting, *B. subtilis* B2G and pseudomonad APF1 and APF2 strains significantly reduced wilt incidence (df = 6, $F = 6.05$, $P < 0.0001$)

(Fig. 5.5a) and severity (df = 6, $F = 7.63$, $P < 0.0001$) (Fig. 5.5b) compared to the control. At 57 days after transplanting only *B. subtilis* B2G and pseudomonad APF1 strains were significantly different from the control for both incidence (df = 6, $F = 4.57$, $P = 0.0007$) and severity (df = 6, $F = 4.21$, $P = 0.013$). Strains *S. setonii* RP87 and pseudomonad APF2 and APF3 did not significantly differ from the control throughout the recorded time. Generally, the most effective strains, pseudomonad APF1 and *B. subtilis* B2G, reduced disease incidence at 45 days after transplanting by 70 and 63% and at 57 days after transplanting by 53 and 52%, respectively. Similarly, pseudomonad APF1 and *B. subtilis* B2G strains reduced disease severity at 45 days after transplanting by 77 and 61% and at 57 days after transplanting by 52 and 50%, respectively.

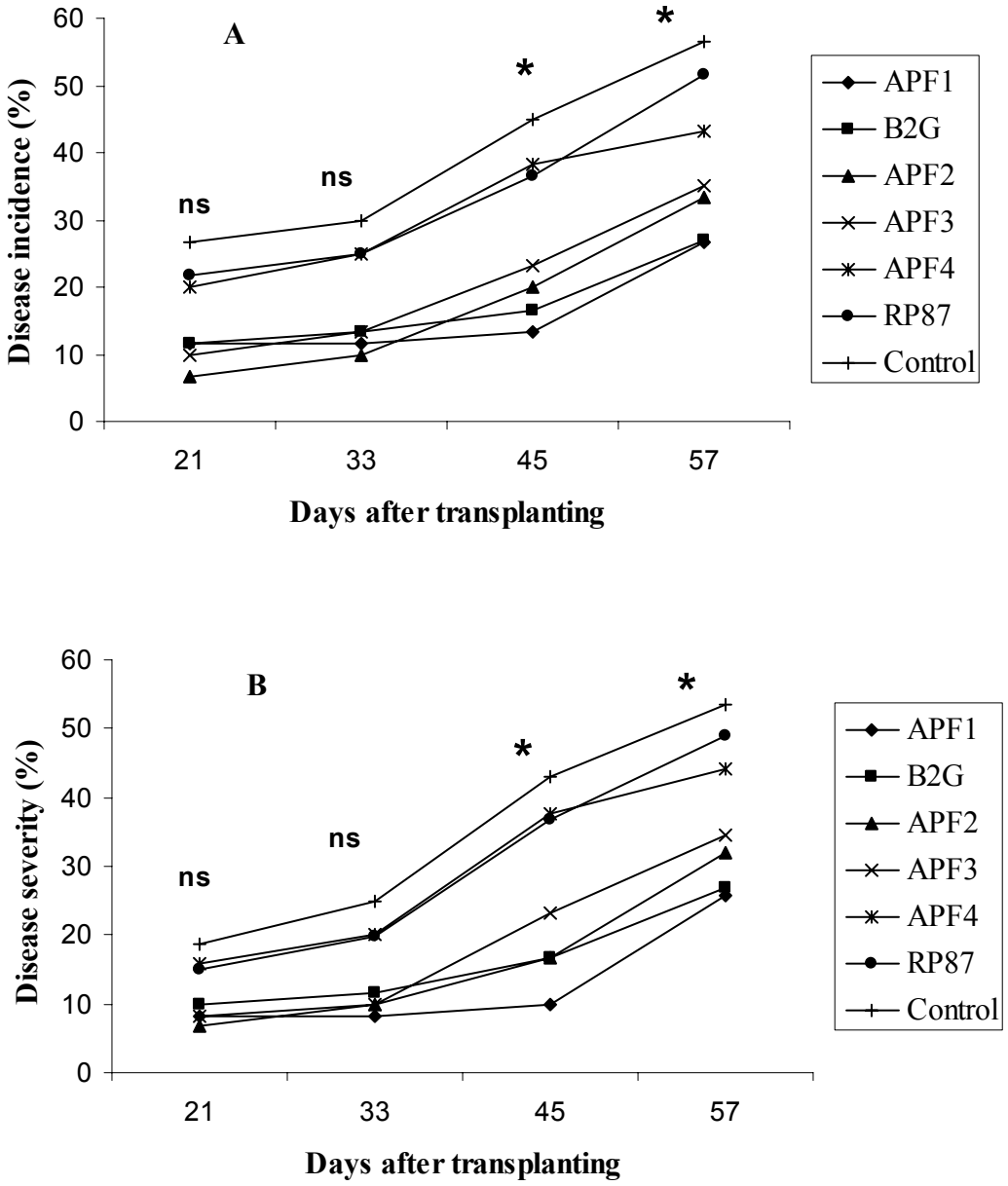


Fig. 5.5. Development of disease incidence (A) and severity (B) on tomato plants treated by different bacterial antagonists (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and Fluorescent pseudomonads APF1, APF2, APF3 and APF4). ns and * indicate absence and presence of significant differences ($\alpha = 0.05$) among treatments at a particular day after transplanting, respectively.

Wilt incidence and severity in the form of AUDPC were also significantly affected by treatments. Plants treated with *B. subtilis* B2G, and pseudomonad APF1 and APF2 strains sustained significantly lower incidence (df = 6, $F = 3.93$, $P = 0.0022$) and severity AUDPC

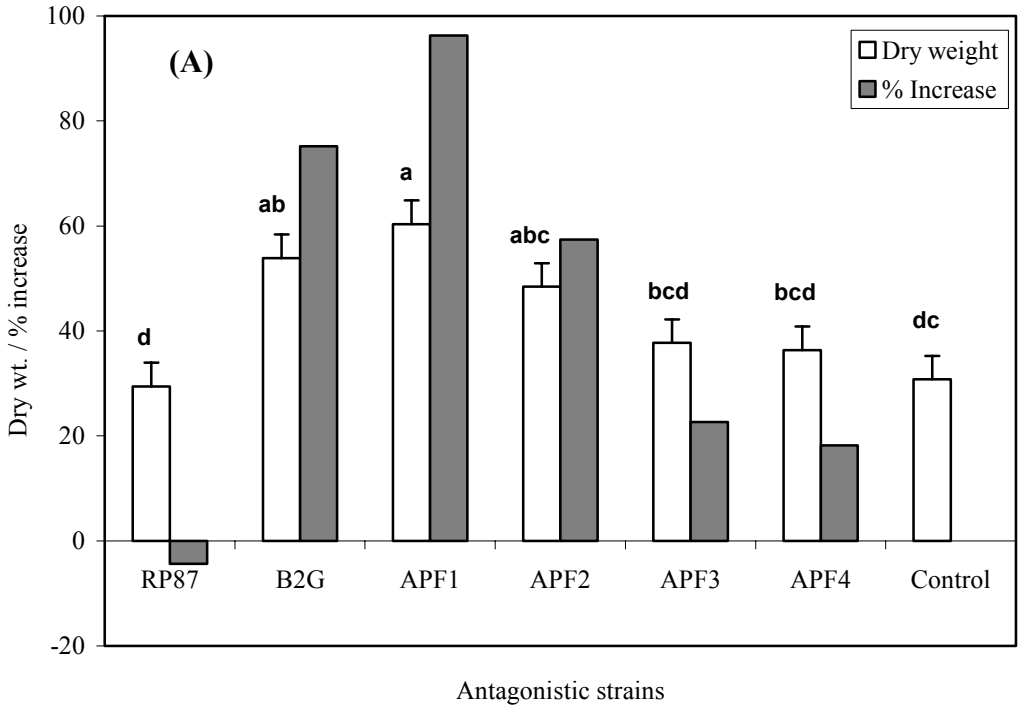
(df = 6, F = 5.20, P = 0.0002) compared to control treatment (Table 5.5). Though the strain *S. setonii* RP87 showed very high inhibition in *in vitro* test, it failed to reduce disease in the *in vivo* biocontrol assay. Generally, no correlation (data not shown) was found between *in vitro* antagonism and *in planta* suppression of disease. Biocontrol efficacy for incidence and severity AUDPC ranged from 12.55 to 60.28% and 13.32 to 65.46%, respectively. The highest biocontrol efficacy was recorded by Pseudomonad APF1 followed by *B. subtilis* B2G and pseudomonad APF2.

Table 5. 5. Effect of antagonistic bacterial strains (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and Fluorescent pseudomonads APF1, APF2, APF3, and APF4), on *Ralstonia solanacearum* wilt incidence and severity expressed as area under disease progress curve (AUDPC) on tomato plant in the greenhouse

Strain*	Incidence AUDPC (%-days)	Biocontrol efficacy (%)	Severity AUDPC (%-days)	Biocontrol efficacy (%)
RP87	983.3 ab	12.6	886.6 ab	13.3
B2G	493.6 b	55.6	467.6 bc	54.3
APF1	441.6 b	60.3	353.3 c	65.5
APF2	499.9 b	50.0	460.3 bc	55.0
APF3	632.6 ab	43.1	589.5 abc	42.4
APF4	949.9 ab	14.5	875.9 ab	14.7
Control	1111.6 a	-	1022.9 a	-

* The root system of 4-month-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 to 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, $\alpha = 0.05$).

Plant weight and growth promotion: There were significant differences among treatments for both dry and fresh weights of tomato plants (Fig.5.6a and b). Average dry weight of tomato was significantly higher (df = 6, $F = 8.20$, $P < 0.0001$) with pseudomonad APF1 and *B. subtilis* B2G and average fresh weight was significantly higher (df = 6, $F = 11.79$, $P < 0.001$) with strains *B. subtilis* B2G and pseudomonad APF1 and APF2. Highest dry and fresh weight increase was recorded with pseudomonad APF1 strain treatment with 96 and 81%, respectively. Dry and fresh weight in *B. subtilis* B2G was by 75 and 54% higher than the control, respectively. No significant difference was observed between control and *S. setonii* RP87 and pseudomonad APF3 and APF4 strains in both dry and fresh weight. Simple correlation analysis showed that there were negative and significant associations between disease AUDPC and tomato dry and fresh weights. Incidence 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. Moreover, severity AUDPC was correlated with fresh and dry weight with correlation coefficients r = -0.59 ($P < 0.0001$) and r = -0.37 ($P = 0.0014$) (n = 70).



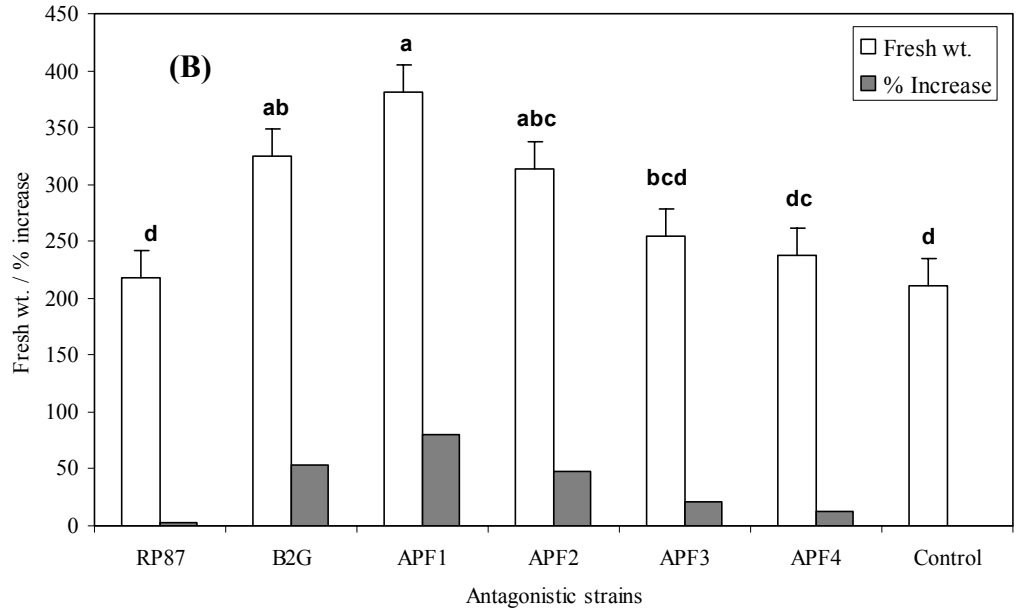


Fig. 5.6. Effect of antagonistic bacterial strains (*Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and Fluorescent pseudomonads APF1, APF2, APF3 and APF4) on dry (A) and fresh (B) weight of tomato plants and per cent increase compared to control. Bars with the same letter are not significantly different according to Tukey's test ($\propto = 0.05$)

The plant growth promotion efficiency of antagonistic isolates monitored by measuring plant biomass (fresh and dry weight) showed variation between plants treated with antagonists and untreated control. There were significantly higher dry (df = 6, F = 1.52, P = 0.0185) and fresh (df = 6, F = 2.57, P = 0.0273) weights of tomato plants treated by pseudomonad APF1 compared with the untreated control (Table 5.2). However, there was no significant difference between control and other antagonistic strains. Treatment with pseudomonad APF1 resulted in 63 and 72% increase in dry and fresh weights of tomato, respectively, compared to the untreated control. Pseudomonad APF2 strain had also increased dry and fresh weight by 24 and 49%, respectively, but the increase was not significant.

Table 5.6. 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

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	-

Values within the same column followed by the same letter do not differ significantly (α = 0.05) according to Tukey's mean separation test.

5.4. Discussion

Root associated bacteria are an important functional group of beneficial bacteria used for control of soilborne pathogens and plant growth promotion (Weller, 1988; Kloepper e*t al.,* 1999; Gamalero *et al.,* 2003; Haas and Keel, 2003; Rajkumar *et al.,* 2005). In this investigation, root associated bacteria were isolated from the rhizosphere of mainly potato, tomato, and pepper with the objective of selecting efficient antagonists against soilborne infection with *Ralstonia solanacearum*, the causal agent of bacterial wilt.

In *in vitro* screening on LB and KB media by chloroform vapour and agar diffusion methods, six strains (*Streptomyces setonii* RP87, *Bacillus 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 5.2). This seemed to be indicative 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 with 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 may be supported by the observation that amendment of KB medium with iron reduced the inhibitory activity of pseudomonad strains APF1 and APF2 (Table 5.4). This is in agreement with other observations that inhibition of growth on KB could be a consequence of production of siderophores by the *Pseudomonas* isolates (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; Notz *et al.,* 2002; Chen *et al.,* 2003). For instance, Duffy and Défago (1999) noticed that addition of zinc increases antibiotic synthesis in pseudomonads. Furthermore, Formica (1990) reported that the type of sugar molecules may change the production of antifungal metabolites. 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.

On the other hand, the inhibitory activity of the strains *Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and pseudomonads APF3 and APF4 was equal in LB and KB (Table 5.2), 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 *Streptomyces setonii* RP87, *Bacillus 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) and Montealegre *et al.* (2003) suggested that when an antagonist is equally effective in the absence and presence of iron, antibiotic production seems to be active against the pathogen. Furthermore, pseudomonads APF1 and APF2 which were producing siderophores, did not loose their inhibitory activity when KB was supplemented with iron, but it was significantly reduced (nearly by 50%) (Table 5.4). Thus it is possible to conclude that pseudomonad APF1 and APF2 strains produce other antagonistic metabolites in addition to siderophores.

The population dynamics studies of *R. solanacearum* in the soil showed that the amount of the 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. 5.4). Later on, 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 other strains and the control, population steadily increased. Of all strains, pseudomonad APF1 strain minimized the population at both 6 and 9 weeks after treatment of soil, while *B. subtilis* B2G minimized only 6 weeks after application. 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 applied to soil from laboratory. Nevertheless, population reduction in soil by pseudomonad APF1 and *B. subtilis* B2G strains seemed to be due to their antagonistic effects.

The tendency of disease development on plants was closely related to population dynamics of *R. solanacearum* in soil (Fig.5.4 and Fig.5.5). Disease progressed slowly at early stage (up to and 33 days after transplanting) and started to progress fast 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 APF1 and APF2 strains. Disease incidence and severity was significantly reduced by pseudomonad APF1 and *B. subtilis* B2G and correlated with a decrease of the population of the pathogen in soil. Similar reports of decline of pathogen in the soil due to antagonists and disease reduction were presented recently (Pieterse *et al.,* 2001; Szczech and Shoda, 2004).

Under greenhouse conditions, the antagonistic isolates pseudomonad APF1 and *Bacillus subtilis* B2G showed a significant reduction of disease compared to the control. Both strains reduced severity AUDPC by 65 and 54% and incidence AUDPC by 60 and 56%, respectively. Moreover, significantly higher amount of biomass (fresh and dry weight) compared to the

control was found by these strains. Guo *et al.* (2004) and Wydra and Semrau (2005) reported comparable *R. solanacearum* wilt disease reduction and yield increase on tomato plants after treatment by *Bacillus* spp. and fluorescent pseudomonads. Moreover, Priou *et al.* (2005) recorded 80% reduction of the same disease on tomato plants under greenhouse conditions using *Pseudomonas putida*. Antagonistic *Pseudomonas* spp. were tested for their ability to suppress bacterial wilt in tobacco and some showed promising results (Liu *et al.,* 1999; Zhang *et al.,* 1999). Recently, Ran *et al.* (2005) also reported suppression of bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. Moreover, Ciampi *et al.* (Website accessed in June 2005), proved the use of antagonistic bacteria *P. fluorescens* BC8 and *B. subtilis* A47 to be much effective in the control of *R. solanacearum* under field conditions and the authors were working on formulation of the two strains for the ease of storage and field use.

There are several modes of action known by the applied rhizobacteria in controlling plant diseases (Hessenmüller and Zeller, 1996; Blanco *et al.,* 2004; Ran *et al.,* 2005; Dwivedi and Johri, 2003). With this respect, the species of *Pseudomonas* and *Bacillus* are probably the best studied groups (Zeller, 1999; Thomashow *et al.,* 1990; Dwivedi and Johri, 2003). Pseudomonads exert a protective effect on the roots through antagonism towards phytopathogenic bacteria by producing metabolites that include: i) biosurfactants e.g., rhamnolipids (Stanghellini and Miller, 1997) and hydrogen cyanide (Schippers *et al.,* 1991; Voisard *et al.,* 1989); ii) lytic enzymes e.g., ß-1,3 glucanase, protease (Friedlender *et al.,* 1993; Berg, 1996); iii) plant hormones and other plant growth promoting substances e.g., auxins, indole-3-acetic acid (Loper and Schroth, 1986), gibberellins (Ramamoorthy and Samiyappan, 2001) and 1-aminocyclopropane-1-carboxylate deaminase (Jacobson *et al.,* 1994); iv) siderophores e.g., pyoverdins, pseudobactin and pyochelin (Leong, 1986; Duijff *et al.,* 1994; Dwivedi and Johri, 2003); v) antibiotics e.g., phenazines (Défago and Haas, 1990;

Thomashow *et al.,* 1990), pyoluteorin (Kraus and Loper, 1995; Dwivedi and Johri, 2003), pyrrolnitrin (Défago and Haas, 1990; Lambert *et al.,* 1987), and 2,4-Diacetyl phloroglucinol (Raaijmakers and Weller, 1998; Harrison *et al.,* 1993; Haas and Keel, 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; Fiddaman and Rossall, 1993) and lytic enzymes (Frändberg and Schnürer, 1994). Some of the antibiotics produced by *Bacillus* spp. include: bacillomycin, mycobacillin, iturin A, surfactin, mycosubtilin, fungistatin, subsporin, bacilysin, chlorotetetain (Krebs *et al.,* 1998; Loeffler *et al.,* 1990; Asaka and Shoda, 1996).

As we did not detect the lytic enzymes cellulase, protease, lipase and levansucrase during our study, it is likely that one or more of the secondary metabolites mentioned above other than the lytic enzymes were responsible for the antagonistic activity towards *R. solanacearum*. In a study made by Montealegre *et al.* (2003), *Bacillus subtilis* and *B. lentimorbus* were effective in controlling *Rhizoctonia solani* on tomato plant without secreting lytic enzymes - glucanase, protease and chitinase. However, Buchenauer (1998) stated that lytic enzymes secreted by bacteria are suspected to play an important role in suppression of pathogens. Production of extracellular lytic enzymes, e.g., ß-1,3-glucanases, lipases, and proteases may be involved in antagonism against an array of plant pathogens (Madsen and de Neergaard, 1999; Foley and Deacon, 1986).

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* was documented also in other studies on other pathogens (e.g., Ran *et al.,* 2005; Ryan, *et al.,* 2004; 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 (Smith and Goodman, 1999, Notz *et al.,* 2002, Ownley *et al.,* 2003; 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 plant fresh and dry weight compared to untreated control by 72 and 63%, respectively (Table 5.6). The plant growth promotion by rhizosphere bacteria might be associated with secretion of auxins, gibberellins and cytokinins (Loper and Schroth, 1986; Jacobson *et al.,* 1994; Ramamoorthy and Samiyappan, 2001) and suppression of deleterious microorganisms in the rhizosphere (Gamliel and Katan, 1993). The use of rhizosphere bacteria for increasing the yield and crop protection is an attractive approach in the modern system of 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 *in vivo* biocontrol assay under greenhouse conditions. Thus future research should be directed towards detailed mode of action of these effective strains and field studies should be undertaken to confirm the effectiveness of the antagonistic isolates under natural conditions in the field.

6 General Discussion

Ralstonia solanacearum is a plant pathogen that causes bacterial wilt on potato and other host plants and is heterogeneous in biochemical, pathogenic and genetic characteristics (Poussier *et al.,* 1999; Hayward *et al.,* 1990; Horita and Tsuchiya, 2001). In order to develop an effctive and sound control measure for such a diverse pathogen, it seems to be important to have prior information on its properties. With this respect, first the biochemical and pathogenic characteristics (Chapters 2 and 3) and genetic diversity (Chapter 4) of Ethiopian *R. solanacearum* strains had been investigated. Moreover, as there has been no available effective control method against the disease, studies on the biocontrol of the pathogen using bacterial antagonists (Chapter 5) have been also carried out with the objective of finding potential biocontrol agents that can be used, if not alone, integrated with other control methods.

Based on biochemical characteristics (i.e. utilization of carbohydrates) and host reaction, Ethiopian *R. solanacearum* strains have been classified into five biovars (Hayward 1964; He *et al.,* 1983; Hayward *et al.,* 1990) and five races (Buddenhagen *et al.,* 1962; Kelman and Person, 1961; He *et al.,* 1983, Horita and Tsuchiya, 2001). Accordingly, the present study (Chapters 2 and 3), grouped Ethiopian *R. solanacearum* strains into biovar I/race 1 and biovar II/race 3. Although previous studies from Ethiopia have reported only biovar II/race 3 strains (Yaynu, 1989), in our studies the existence of biovar I/race 1 strains in Ethiopian *R. solanacearum* population could be detected. Berga *et al.* (1994) indicated previously that, large ammounts of potato tubers from different parts of the world, mainly from the International Potato Center in Peru, South America, have been introduced into Ethiopia in order to improve resistance and productivity of the local potato germplasm. Thus, as biovar I

race 1 strains are known to be widely distributed throughout tropical areas (He *et al.,* 1983; Buddenhagen *et al.,* 1962), it may be possible that they have been introduced to Ethiopia via latently infected tubers from other regions of the world, as dissemination of the bacterium is mainly through latently infected planting materials (Hayward, 1991; Williamson *et al.,* 2002).

The present study showed also that the response of biovars to different regimes of temperature is variable (Chapter 2). In consequence, biovar II strains were able to grow to high density at lower temperature (22°C), while biovars I and III strains at higher temperatures (32 and 37°C). Thus it could be shown that biovar II strains prefer lower temperature, while biovars I and III higher temperature. This supports the assumption that biovar II and biovars I and III are more adapted to cool and warm tropical conditions (Horita *et al.,* 2005; Swanson *et al.,* 2005; Marin and El-Nashaar, 1993; French *et al.,* 1993). However, it seems that the strains can survive and cause infection out of their area of adaptation too. Because during this study there were strains collected out of their range of adaptation. For instance, biovar I strains Pot 46 and Pot 48 were isolated from potato plants grown at higher altitudes of 2600 and 2200 meters above sea level, respectively, in Ethiopia and a Peruvian biovar I strain GSPB 2791 was originally from 2000 meters above sea level. French *et al.* (1993) has reported similar cases and it is assumed to be due to distribution of infected potato tubers from one area to the other.

Differentiation of *R. solanacearum* into races is based on pathogenicity test with the age of inoculation at 3 to 4 true leaf stage (Hayward 1991; Horita and Tsuchiya, 2001; OEPP/EPPO, 2004). When inoculation was carried out at different ages (1 to 2, 3 to 4 and 5 to 6 true leave stages) of hosts in order to know its effect on disease development, age of inoculation affected disease development depending on type of host and race of the pathogen. For instance, in potato, tomato and eggplant, disease was not affected by age of inoculation and type of race.

However, in pepper and tobacco, disease development by virulent strain (race 1) was significantly reduced when inoculation was made at 4-5 true leave stage as compared to the inoculation at earlier stages. This indicates that if the objective of a study is to determine race of a pathogen, delayed inoculation (compared to the standard 3 to 4 leaf stage), may lead to a wrong conclusion in race determination as pepper and tobacco plants remain healthy as if they are resistant. On the other hand, the fact that pepper and tobacco remained healthy when inoculated with virulent strain at delayed stage of inoculation may indicate that the resistance in pepper and tobacco is related to age. Although not against *R. solanacearum*, against some plant pathogens, pepper (Hwang, 1995) and tobacco (Kus *et al.,* 2002; Yalpani *et al.,* 1993) resistance have shown positive correlation with age which was due to accumulation of capsidiol and salicylic acid, respectively.

The results of biochemical (Chapter 2) and pathogenicity tests (Chapter 3) in this study classified Ethiopian strains into two main groups of biovars (I and II) and races (1 and 3), respectively. Similarly, results of genetic diversity study with rep-PCR (Chapter 4) revealed the existence of two main groups in Ethiopian *R solanacearum* strains. With the dendrogram drawn from the rep-PCR analysis, group one embraced all biovar II/ race 3 strains, while group 2 included all biovar I/race 1 strains at 55% similarity level. Furthermore, the PCR analysis revealed that Ethiopian strains can be grouped into five subgroups at 90% similarity level. Generally, however, Ethiopian biovar II strains were found to be more diverse than biovar I strains. This is as opposed to many reports which, with investigations on *R. solanacearum* strains collected from different parts of the world (Africa, Asia, Australia, America and Europe), stated that biovar II are the most homogeneous group (e.g., Smith *et al.,* 1995; Poussier *et al.,* 1999). The disagreement between ours and previous findings may be due to genetic changes in strains over time that could contribute for diversity of the population

or it may be due to the fact that the world population of *R. solanacearum* strains were not well represented in the previous studies.

The cluster analysis in Chapter 4 showed that Ethiopian biovar I strains are homogeneous as they were pooled in one group at 90% similarity level. However, biovar I strains are known to be the most diverse population of *R. solanacearum* (Poussier *et al.,* 1999; Smith *et al.,* 1995; Horita and Tsuchiya, 2001). In the present study (Chapters 2 and 3) it was found out that biovar I/race 1 is a new report from Ethiopia. Thus the homogeneity of Ethiopian biovar I/race 1 strains as opposed to reports by other authors mentioned before could be due to narrow genetic basis of biovar I strains introduced to Ethiopia.

Genetic diversity studies by RFLP and other DNA-based techniques on *R. solanacearum* strains collected from different countries in Africa, Europe, Asia, Australia and America have revealed the presence of two main divisions (Cook *et al.,* 1989; Taghavi *et al.,* 1996). Division I (Asiaticum) comprised all members of biovars III, IV, and V, while division II (Americanum) comprises biovars I and II. Similarly, our cluster analysis divided the studied strains into two groups at 25% similarity level. Accordingly, all Ethiopian strains were found to be American origin. The fact that Ethiopia had introduced potato genotypes mainly from Peru, South America, for improving the local cultivars (Berga *et al.,* 1994) supports the assumption that latently infected potato tubers are the main means of *R. solanacearum* dissemination (Hayward, 1991).

Results from Chapter 5 clearly indicate that *in vitro* inhibition effect of antagonists on *R. solanacearum* depends on type of media used. For instance, among tested antagonistic strains, fluorescent pseudomonad APF1 and APF2 strains showed significantly higher inhibition in KB medium than in LB. However, in *Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and

pseudomonad APF3 and APF4 strains, inhibition of *R. solanacearum* was the same in both media. As KB medium is deficient in iron (Lim and Kim, 1997) increase of inhibition in KB medium by APF1 and APF2 strains was attributed to siderophore production. This was confirmed by supplementing KB medium with iron which led to significant reduction of inhibition of *R. solanacearum*. Inhibition of growth in KB medium has been reported to be related to siderophore production by *Pseudomonas* spp. (Blanco *et al.,* 2004; Lim and Kim, 1997). In *Streptomyces setonii* RP87, *Bacillus subtilis* B2G, and pseudomonad APF3 and APF4 strains as there was no inhibition difference between iron deficient and iron supplemented KB media, the inhibition could be due to antibiotic production. Ran *et al.* (2005) has indicated that equal effectiveness in iron supplemented and deficient KB medium shows antibiotics playing active role.

Under greenhouse conditions, among all strains tested fluorescent pseudomonad APF1 and *B. subtilis* B2G strains consistently reduced wilt disease and increased weight of tomato plant. Moreover, they induced a reduction of the pathogen in the soil which might partly contribute for disease reduction and weight increase. There were reports in other host-pathogen combinations where decline of pathogen in the soil due to antagonists was related with a reduction of disease (Pieterse *et al.,* 2001; Szczech and Shoda, 2004).

Several mechanisms have been proposed for suppression of pathogens by *Pseudomonas* and *Bacillus* spp. (Ran *et al.,* 2005; Hessenmüller and Zeller, 1996; Blanco *et al.,* 2004; Dwivedi and Johri, 2003). Pseudomonads provide a protective effect on the roots through antagonism towards phytopathogenic bacteria by producing metabolites such as: biosurfactants e.g., hydrogen cyanide (Schippers *et al.,* 1991; Voisard *et al.,* 1989), rhamnolipids (Stanghellini and Miller, 1997); lytic enzymes e.g., ß-1,3 glucanase, protease (Friedlender *et al.,* 1993; Berg, 1996); plant hormones and other plant growth promoting substances e.g., auxins,

indole-3-acetic acid (Loper and Schroth, 1986), gibberellins (Ramamoorthy and Samiyappan, 2001) and 1-aminocyclopropane-1-carboxylate deaminase (Jacobson *et al.,* 1994); siderophores e.g., pyoverdins, pseudobactin and pyochelin (Leong, 1986; Duijff *et al.,* 1994; Dwivedi and Johri, 2003); antibiotics e.g., phenazines (Défago and Haas, 1990; Thomashow *et al.,* 1990), pyoluteorin (Kraus and Loper, 1995; Dwivedi and Johri, 2003), pyrrolnitrin (Défago and Haas, 1990; Lambert *et al.,* 1987), and 2,4-Diacetyl phloroglucinol (Raaijmakers and Weller, 1998; Harrison *et al.,* 1993; 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 (Fiddaman and Rossall, 1993; Parke and Gurian-Sherman, 2001) and lytic enzymes (Frändberg and Schnürer, 1994). Some of the antibiotics produced by *Bacillus* spp. include: bacillomycin, mycobacillin, iturin A, surfactin, mycosubtilin, fungistatin, subsporin, bacilysin, chlorotetetain (Asaka and Shoda, 1996; Krebs *et al.,* 1998; Loeffler *et al.,* 1990).

The lytic enzymes protease, lipase, cellulase, and levansucrase were not detected during our study. Therefore, it is likely that one or more of the secondary metabolites mentioned above were responsible for the antagonistic activity towards *R. solanacearum*. In a study made by Montealegre *et al.* (2003), *B. subtilis* and *B. lentimorbus* were effective in controlling *Rhizoctonia solani* on tomato plant but they were not secreting lytic enzymes - glucanase, protease and chitinase. However, Buchenauer (1998) stated that lytic enzymes secreted by bacteria are suspected to play an important role in suppression of pathogens. Production of extracellular lytic enzymes, e.g., ß-1,3-glucanases, lipases, and proteases may be involved in antagonism against an array of plant pathogens (Madsen and de Neergaard, 1999; Foley and Deacon, 1986).

In conclusion, the present study reveals two distinct groups of *R. solanacearum* strains in Ethiopia which are different in biochemical, pathogenic and genetic characteristics. Therefore it seems to be important that control strategies need to be designed taking the available diversity into consideration. Moreover, pseudomonad strain APF1 and *B. subtilis* B2G have been determined as potential antagonists that should be tested under field conditions in order to confirm their efficacy.

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Declaration

I hereby declare that the Ph.D. thesis entitled: **"Biochemical, pathological and genetic characterization of strains of *Ralstonia solanacearum* (Smith) from Ethiopia and biocontrol of *R. solanacearum* with bacterial antagonists"** submitted to the University of Hannover, Germany, is an independent work carried out by me and the thesis has not formed previously the basis for the award of any degree.

Sincerely,

Fikre Lemessa Ocho