Characteristics of *Ralstonia Solanacearum* Strains of Potato Wilt Disease from Nepal and Thailand[1]

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Abstract

Characterization of strains of *Ralstonia solanacearum*, the causal agent of potato bacterial wilt disease from Nepal and Thailand was performed based on pathogenicity, biochemical/physiological and serological tests. Fifteen *R. solanacearum* strains isolated from wilt infected potato plants and tubers grown in Nepal were characterized as race 3, biovar II based on the pathogenicity on different host plants, hypersensitive reaction on tobacco leaf and utilization of some sugars. Results of pathogenicity test show that all strains from Nepal had limited host range. Degree of virulence of all strains varied from high to medium in potato and tomato and medium to low in eggplant. They did not cause wilting in tobacco, pepper and peanut plants. Six strains from Thailand were characterized as biovar II and III. Additionally, comparisons on the physiological, biological and serological characters of seven strains from Nepal and six from Thailand revealed similar characters. Race 3 and biovar II of the pathogen was widely spread over potato growing areas of mid and high hills of Nepal. Both biovars II and III were prevalent in the potato growing areas of Thailand but biovar III was the most dominating one.

Key words: Bacterial wilt, potato, *Pseudomonas solanacearum*, *Ralstonia solanacearum*

Introduction

Ralstonia solanacearum (Yabuuchi et al., 1995), wilt of potato and solanaceous crops including other host plants is formerly known as *Pseudomonas solanacearum* EF Smith. The pathogen is also identified as *Burkholderia solanacearum* (Yabuuchi et al., 1992). Bacterial wilt is one of the most important and widespread diseases of solanaceous plants in the world. In Nepal, the disease is considered as the most important one that causes a considerable yield loss every year (Pradhanang et al., 1993).

Buddenhagen et al. (1962) divided the pathogen into three races. Race 1 infects many solanaceous plants such as tomato, tobacco, pepper and other plants including some weeds. However, race 2 causes wilt of triploid banana (*Musa* spp.) and *Heliconia* spp. Race 3 affects potato and tomato but is weakly virulent on other solanaceous crops. Later, Aragaki and Quinon (1965) reported race 4 from infected ginger in the Philippines. He et al. (1983) reported race 5 from mulberry in China. Therefore, five races have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions (French, 1986).

The four biovars of *R. solanacearum* have been characterized on the basis of utilizing and/or oxidizing three hexoses mannitol, dulcitol and sorbitol and three disaccharides lactose, maltose and cellobiose. Biovar I oxidizes hexose alcohols but not disaccharides, whereas biovar II oxidizes only disaccharides. Biovar III oxidizes both disaccharides and hexose alcohols, but biovar IV oxidizes only alcohols (Hayward, 1964). However, races and biovars are poorly correlated except for race 3, which is more or less similar to biovar II (French, 1986). In Nepal, Shrestha (1977) and Adhikari (1993) reported the race 3 and the biovar II in the potato from mid to high

[1] A part of MSc thesis submitted by the first author to the Kasetsart University, Bangkok, Thailand in 1997 for partial fulfillment of the degree.

hill region and the race 1 and biovar III from eggplant, pepper, tomato and marigold from lowland areas. Titatarn (1986) classified the bacterial wilt pathogen of potato as biovar III and IV from mid hills and biovar II from high hills of Thailand. The objective of this study was to characterize the potato bacterial wilt strain of *R. solanacearum* from Nepal and Thailand based on pathogenicity, biochemical/physiological and serological tests.

Materials and Methods

Bacteria isolation and cultures

Infected potato stems or tubers collected from different sources and locations in Nepal and Thailand (Table 1) were cut into small pieces and placed in test tubes containing 5 ml of sterile distilled water for standard isolation (Hildebrand et al., 1988). Bacteria were allowed to flow from the vascular bundles for 5 to 10 minutes. One loopful of the bacterial suspension was streaked onto tetrazolium chloride (TZC) agar medium (Kelman, 1954) and incubated at 28^{o}C for 48 h. A single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the center was selected and multiplied in a TTC (without adding TZC) medium. After 24-48 h of incubation, virulent cultures were maintained in sterile distilled water in screw-capped tubes at room temperature.

Table 1. Biovar characterization of *Ralstonia solanacearum* strains isolated from bacterial wilt infected potato plants in Nepal and Thailand

Strain	Location	Saccharides reactions†						Biovar classification
		Maltose	Lactose	Cellobiose	Mannitol	Sorbitol	Dulcitol	
Nepal								
NSPC 1	Nucleus Seed Potato Center, Sindhupalcok	+	+	+	-	-	-	II
NSPC 3	Nucleus Seed Potato Center, Sindhupalcok	+	+	+	-	-	-	II
NF 5	Nigale, Sindhupalcok	+	+	+	-	-	-	II
NF 6	Nigale, Sindhupalcok	+	+	+	-	-	-	II
MB 9	Mude, Sindhupalcok	+	+	+	-	-	-	II
MB 10	Mude, Sindhupalcok	+	+	+	-	-	-	II
MB 12	Mude, Sindhupalcok	+	+	+	-	-	-	II
KD 17	Kharidhunga, Dolakha	+	+	+	-	-	-	II
BA 2	Balaju, Kathmandu	+	+	+	-	-	-	II
BA 4	Balaju, Kathmandu	+	+	+	-	-	-	II
BA 5	Balaju, Kathmandu	+	+	+	-	-	-	II
SA 1	Sankhu, Kathmandu	+	+	+	-	-	-	II
SA 2	Sankhu, Kathmandu	+	+	+	-	-	-	II
NA 4	Nala, Kavrepalchok	+	+	+	-	-	-	II
NA 5	Nala, Kavrepalchok	+	+	+	-	-	-	II
Thailand								
1073	Doi Poo Muan, Fang, Chiang Mai‡	+	+	+	+	+	+	III
1089	Hort. Res. Station, Kaoko, Petchabun‡	+	+	+	+	+	+	III
1155	Hueysithon, Fang, Chiang Mai‡	+	+	+	-	-	-	II
1252	Noungmaloa, , Lumpun‡	+	+	+	-	-	-	II
1255	J.D. Kok, Mae Sot Tak‡	+	+	+	+	+	+	III
KUT 1	Kasetsart market, Bangkok	+	+	+	+	+	+	III

† + Positive reaction (color of medium was changed from green to yellow); - Negative reaction (color of medium was not changed); ‡ Received from Mr. W. Boenjuebsaku, Bacteriology Section, Department of Agriculture, Bangkok.

Hypersensitive reaction and pathogenicity test

All fifteen bacterial wilt strains of potato from Nepal were tested for hypersensitive reaction (HR) on tobacco leaf (Table 2). The bacterial suspension was prepared and adjusted to 0.2 OD (optical density) at 600 nm by Spectonic 20 (Bausch and Lomb, Co. Ltd.), which was about 10^8 colony forming unit (cfu) per ml. One side of completely expanded tobacco leaves was infiltrated with 1.0 ml of bacterial suspension and the opposite sides with water as a control. The HR was observed daily for 5 days after infiltration of bacterial suspension.

Table 2. Pathogenicity test on potato, tomato and egg plant and hypersensitive reaction (HR) on tobacco leaves and classification of *Ralstonia solanacearum* strain in Nepal

Strain	Pathogenicity reaction†			HR‡
	Potato	Tomato	Egg plant	
NSPC 1	M	H	L	+
NSPC 3	H	H	M	+
NF 5	M	H	M	+
NF 6	M	M	L	+
MB 9	M	M	L	+
MB 10	M	M	L	+
MB 12	H	H	L	+
KD 17	M	M	L	-
BA 2	M	H	L	+
BA 4	H	M	M	+
BA 5	M	M	L	+
SA 1	M	M	L	-
SA 2	M	M	M	+
NA 4	M	M	M	+
NA 5	M	M	L	+

† Average disease indices of 5 plants at 28 days after inoculation and rating scales (He et al., 1983) were as followed: H, High (disease index 4.1 to 5.0); M, Moderate (2.6 to 4.0); L, Low (1.1 to 2.5); and, 0, None (1.0).
‡ + Infiltrated area become necrosis; - No reaction.

A mixture of substrates eg sand, compost and soil (1:1:1) treated with formalin was prepared and filled in clay pots of 20 cm diameter. Six different host plants, such as tobacco (*Nicotiana tabacum* L. cv Local), tomato (*Lycopersicon esculentum* Mill. cv Pussa Ruby), eggplant (*Solanum melongena* L. cv Nurkee), pepper (*Capsicum annuum* L. cv California Wonder), potato (*Solanum tuberosum* L. cv Kufri Jyoti) and peanut (*Arachis hypogaea* L. cv Local) were planted in the pots and placed in glasshouse at Khumaltar, Nepal. All test plants were allowed to grow for 6-8 weeks or until they were 15-20 cm high. Five plants of each host were inoculated with each strain of the bacterium by inserting a sterile micropipette tip containing 100 µl at the axil of a fully expanded leaf from the top. The micropipette tips were left in position until the inoculum was absorbed. Inoculated plants were observed daily for evaluation of pathogenicity and severity. Disease severity was assessed at weekly interval for four weeks following the scale of He et al. (1983) (1, no symptoms; 2, two leaves wilted; 3, three leaves wilted; 4, four or more leaves wilted and 5, plant dead).

Physiological and biochemical test

Seven bacterial strains of potato wilt from Nepal and six from Thailand (Table 1) were characterized by using the following tests: oxidation/fermentation, starch hydrolysis, indole production and nitrate (NO_3) reduction (Hayward, 1964; Lelliott and Stead, 1987; Hildebrand et al., 1988). Additionally, the tests such as oxygen relation, levan production, urease test, gelatin liquefaction, tween 80 hydrolysis, catalase production, sodium chloride (5 and 7%) tolerance, oxidase test and growth on potato slice were also performed according to Lelliott and Stead (1987), Hildebrand et al. (1988). Furthermore, some tests were made on arginine dihydrolase, motility, citrate utilization and ammonia production following the method of Hildebrand et al. (1988).

Biovar characterization was carried out based on the ability of strains to oxidize certain disaccharides and sugar alcohols as described by Hayward (1964). Dulcitol, mannitol, sorbitol, cellobiose, lactose and maltose were prepared at 10% solution in distilled water and added separately into Hayward's basal medium modified by He et al. (1983) in order to make a final concentration 0.1%. Each medium was inoculated separately with one loopful of 48 h old bacterium culture of each strain and the tubes containing such cultivars were incubated at room temperature up to 30 days.

Serological test

Seven potato strains of *R. solanacearum* from Nepal and six from Thailand, were tested by using immunofluorescence (IF) test (Schaad, 1978) against an antiserum produced from whole cells of *R. solanacearum* from ginger. Suspension of one loopful of each culture was made in 1.0 ml NaCl (0.85%) with 100 µl of sterile formalin (40%) solution. After mixing, 5 µl of each suspension was placed on each well of a multiwells slide and replicated two times. The slides were air-dried and flooded with Kirkpatrick's fixative (ethanol 60%, chloroform 30% and formalin 10%) and kept in a petri dish with moist filter paper for 5 minutes. The slides were rinsed with fixative and allowed to dry. After drying, the slides were stained with *R. solanacearum* antiserum and the control wells were treated with 0.01 M phosphate buffered saline (PBS) and then incubated in a moist

chamber in the dark for 30 minutes. The slides were rinsed with sterile NaCl solution, followed by PBS buffer and sterile distilled water and then dried. Again slides were stained with a second antiserum (goat antirabbit IgG conjugated FITC fluorescent dye) and placed in a moist chamber in the dark for 20 minutes. The slides were rinsed and dried as described above. Finally, the slides were mounted in a carbonate buffered solution (pH 9.0) with glycerin and examined under the epifluorescent microscope.

Results and Discussion

Pathogenicity test and hypersensitive reaction

All fifteen potato strains of *R. solanacearum* from Nepal produced fluidal and irregular colonies with pink or light red at centers on TZC medium at 30^{o}C after 48 h of incubation. Thirteen strains out of fifteen caused necrosis in tobacco leaves within three days of infiltration (Table 2). Two strains, SA-1 and KD-17 showed slow collapses after five days of infiltration. Among fifteen strains tested on differential hosts, NSPC-3 and MB-12 were highly virulent on potato and tomato plants but were moderate to slight virulent on eggplant after four weeks of inoculation. Similarly, the degree of virulence of the strain BA-4 was high in potato and moderate in both tomato and eggplant. Other strains NSPC-1, NF-5 and BA-2 were highly virulent on tomato and moderate to low on both potato and eggplant. The rest of the strains showed moderate to low virulence on potato, tomato and eggplant. None of the strains expressed wilting symptom in tobacco, pepper and peanut. Therefore, all of them had characteristic of race 3 with a limited host range on potato, tomato and a few other hosts (Table 2).

In a host range study, all strains were pathogenic (low to high) on potato, tomato and eggplant. But other hosts such as tobacco, pepper and peanut did not show wilting symptoms. The limited host range is the characteristic of race 3 of *R. solanacearum* (Buddenhagen et al., 1962; He et al., 1983; French, 1986).

Physiological and biochemical tests

All thirteen strains were arginine dihydrolase negative and oxidase, catalase and urease positive. All of them oxidized citrate within 4-5 days of inoculation by changing blue media into green. On the other hand, none of the strains either hydrolyzed starch or produced indole and liquefied gelatin. Strains from Nepal and Thailand were highly sensitive to NaCl at 5% but not at 7%. All the strains produced nitrate and ammonia after 2-3 days of inoculation and they showed positive reactions in levan production, motility, lipolytic and oxygen relation. These strains also hydrolyzed tween 80 and produced black color in potato slants (Table 3).

Biochemical test of all 15 bacterial wilt strains from Nepal oxidized disaccharides, maltose, lactose and cellobiose by changing color of the medium from green to yellow. On the other hand, the strains failed to oxidize hexose sugar alcohols; mannitol, sorbitol and dulcitol, even after 28 days of inoculation (Table 1). Therefore, they were classified as biovar II. Four out of six strains from Thailand oxidized three disaccharides and three hexose sugar alcohols, which were the characteristics of biovar III. However, two strains of *R. solanacearum* 1155 and 1252, oxidized disaccharides but failed to oxidize hexose sugar alcohol even after 28 days of incubation. Such were the characteristic of biovar II (Table 1).

Serological test

Seven strains from Nepal and six strains from Thailand showed positive fluorescent staining by IF test but the strain BA 4 from Nepal showed moderate fluorescence, whereas the control, *Bacillus* spp. (non pathogen) was negative (Table 4). Similarly, strains 1073, 1089 and KUT 1 from Thailand produced moderate fluorescence. From the IF test, it was concluded that all strains were positive to the antiserum from ginger but *Bacillus* sp. was negative. This confirmed the serological character of *R. solanacearum*.

Table 3. Biochemical and physiological characteristics of *Ralstonia solanacearum* from wilted potato plant in Nepal and Thailand compared with strains from China

Biochemical/ Physiological test	Reactions†													
	Nepal strains							Thailand strains						China
	NSPC3	NF6	MB10	KD17	BA4	SA1	NA4	1073	1089	1155	1252	1255	KUT1	strains‡
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Levan production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Uerase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidative	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentative	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on potato	+	+	+	+	+	+	+	+	+	+	+	+	+	NA
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	NA
Ammonia production	+	+	+	+	+	+	+	+	+	+	+	+	+	NA
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	NA
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salt tolerance at 5%	+	+	+	+	+	+	+	+	+	+	+	+	+	NA
Salt tolerance at 7%	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
Oxygen relationship	+	+	+	+	+	+	+	+	+	+	+	+	+	NA
Hydrolysis of Tween 80	+	+	+	+	+	+	+	+	+	+	+	+	+	NA

† + Positive reaction or growth; - Negative reaction or no growth; +/- Reaction not defined.
‡ NA, Not available.

Table 4. Immunoflurescent (IF) test of *Ralstonia solanacearum* strains from wilted potato plant in Nepal and Thailand by using antiserum of *Ralstonia solanacearum* from diseased plant of ginger

Strain	IF reaction†
NSPC 3	+++
NF 6	+++
MB 10	+++
KD 17	+++
BA 4	++
SA 1	+++
NA 4	+++
1073	++
1089	++
1155	++
1252	+++
1255	+++
KUT 1	+++
Bacillus sp.	++

† +++ Positive reaction (strong fluorescent); ++ Positive reaction (weak fluorescent); - Negative reaction (no fluorescent).

On the basis of the cultural characters, pathogenicity, physiological/biochemical and serological tests, all strains of *R. solanacearum* from mid and high hills of Sindhupalchok and Kathmandu valley, Nepal and mid hills of Thailand were similar to the strains from other parts of the world (Buddenhagen et al., 1962; Hayward, 1964; He et al., 1983). All strains of *R. solanacearum* from Nepal were confirmed to be race 3 and biovar II. This result also supports the findings of Shrestha (1977) and Adhikari (1994). They also found that biovar II and race 3 were widely distributed to the mid and high altitude of Nepal.

Similarly, out of six strains tested from Thailand, two were characterized as biovar II and four were biovar III. This result also supports the findings of Titatarn (1986). It was reported that biovar II, III and IV were spread over potato growing areas of Thailand (Titatarn, 1986).

The isolated *R. solanacearum* pathogen from Nepal and Thailand was confirmed as the causal agent of bacterial wilt of potato by performing hypersensitivity, physiological/biochemical, pathogenicity, cultural and serological tests. Race 3 and biovar II of the pathogen were widely spread over potato growing areas of mid and high hills of Nepal. It was concluded that pathotypes and biotypes of bacterial wilt pathogens of potato were remained the same in Nepal from the last two decades. Both biovar II and biovar III of *R. solanacearum* were prevalent in Thailand but biovar III was the most dominating one in the potato growing areas of the country.

Acknowledgements

We wish to express our sincere thanks to Nepal Agricultural Research Council (NARC) and Swiss Development Cooperation/Nepal for their

financial and physical assistance. We also wish to extend our appreciation to Mr W Boenjuebsaku, Plant Bacteriology Section, Department of Agriculture, Thailand for providing strains of *R. solanacearum* from Thailand and Mrs P Thammakijjawat, Plant Pathology Department, Kasetsart University, Thailand for her help during laboratory works in Thailand.

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