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Bitter Gourd (*Memordica charantia* L.), New Host of *Ralstonia solanacearum* in Sri Lanka

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Abstract: Bacterial wilt of bitter gourd, caused by *Ralstonia solanacearum*, is endemic in most bitter gourd growing areas of Sri Lanka, causing loss in yield. Control measure requires definite information on associate pathogen, race and biovar characteristics of the pathogen in those endemic areas. Soil samples were collected from mid country areas in Sri Lanka known for high incidence of bitter gourd bacterial wilt. Isolations were performed on triphenyl tetrazolium chloride (TZC) media. Morphological characterization of the pathogen was through simple staining, streaming and KOH solubility test. Molecular confirmation of pathogen's identity was done by PCR using specific primers RALS-F (5'-GAACGCCAACGGTGCGAACT-3'); and RALS-R (5'-GGCGGCCTTCAGGGAGGTC-3') which amplify a fragment of 400 bp size. Race was determined through hypersensitive reaction on tobacco and capsicum leaves and biovar characterization was through carbohydrate utilization test. Twelve bacterial isolates showed the characteristic creamy white colour on TZC medium. The isolates also amplified at 280 bp, confirming the pathogen as *Ralstonia solanacearum*. All isolates belonged to Race 3 and among them four isolates belongs to Biovar V and five isolates belongs to Biovar III while three isolates were undetectable. According to the varietal response against different isolates of *Ralstonia solanacearum* based on hypersensitive reaction, one of the most popular bitter gourd variety of Maya which recognized as susceptible to bacterial wilt, was highly susceptible for the all *Ralstonia solanacearum* isolates. Variety of Matale Green (MG) which recognized as moderately resistant to bacterial wilt was moderately resistant to some isolates and susceptible to other isolates. Variety TIA x MG and MG x IF6 also show varied resistance levels to different isolates of *Ralstonia solanacearum*. The findings are relevant while devising a more targeted management approach to bacterial wilt of bitter gourd in mid country areas of Sri Lanka.

Keywords: Bitter gourd, Bacterial wilt, *Ralstonia solanacearum*, Biovar, Race.

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1. Introduction

Bitter gourd cultivation in Sri Lanka has suffered heavily in the recent past are to the emergence of wilt disease. A mild infection of this wilt problem was noticed in some location in the central province in year 2010. However it becomes a serious problem now casing economic losses to growers in mid country areas. At present this disease has reached epidemic levels in most the bitter gourd growing areas. Fully expanded leaves may also wilt. A section of the pseudo-stem showed brown-reddish discoloration within and along vascular elements. The whole plant collapses and dies, while there are no apparent symptoms on mature fruits. Bacterial ooze (white spontaneous streaming of bacterial slime) might be observed a few minutes after placing cross section of stems into clear water, indicating infestation of vascular bundles by the bacteria. According to these symptoms this pathogen is suspected as *Ralstonia solanacearum.*

Ralstonia solanacearum is a soil borne bacterial pathogen and a major limiting factor in the production of many crop plants around the world (Agrios, 1997). This organism is the causal agent of brown rot of potato, bacterial wilt or southern wilt of tomato, tobacco, eggplant and some ornamentals and moko disease of banana (Stevenson *et al.*, 2001). As a highly diversified pathogen, *R. solanacearum* is considered a species complex, a heterogeneous group of related strains (Fegan and Prior, 2005). There are hundreds of genetically distinct isolates of *R. solanacearum*.

Historically, the species complex was subdivided into races based very loosely on host range, and into biovars based on ability to produce acid from a panel of carbohydrates (Denny and Hayward, 2001). Further classification of *R. Solanacearum* strains from wilt-endemic areas of the world listed strains belonging to race 1 biovar 1 (R1B1) as the most virulent. In 2002, the subgroup of *R. Solanacearum* known as race 3 biovar 2 (R3B2) was listed in the United States as a bioterrorism select agent (Lambert, 2002).

The recognition of the hypersensitive reaction induced by bacterial plant pathogens (Klement and Lovrekovich 1961, 1962; Klement *et al.*, 1964) has stimulated renewed interest in research on host resistance mechanisms for bacteria during the past decade. Klement and Goodman (1967) related progress in studies on hypersensitivity to the status of research on defense mechanisms against bacteria. The hypersensitive reaction has become an extremely useful tool in determination of potential pathogenicity of bacterial isolates (Lelliott *et al.*, 1966), race identification (Lozano and Sequeira, 1970 a) and in related studies on resistance.

Bacterial populations in resistant hosts decline at a time when rapid multiplication is continuing in the susceptible host (Allington and Chamberlain 1949; Chamberlain 1956, 1962; Diachun and Troutman 1964; Omer and Wood 1969; Scharen 1959; Schroth, Vitanza and Hildebrand 1971). However, the correct identification of pathogen and interaction with host cultivars is relevant and necessary for the development of wilt management practices.

2. Methodology

2.1 Collection of wilt affected bitter gourd plants and bacterial isolation

Infected bitter gourd plants with typical symptoms of bacterial wilt were collected from different parts of the mid country area. These disease infected plants was diagnosed initially by visual symptoms and using ooze test (Hayward, 1994). The wilted samples were washed under running tap water to remove soil particles. The stem cuts were surface sterilized with 70% alcohol and cut ends of each wilted sample were dipped into sterile water in test tubes for bacterial ooze.

The bacterial suspension was then streaked on SPA (Sucrose Peptone Agar) media and incubated at 28^0C for 24 hours for the growth of the bacterium.

All the bacterial isolates were purified by streaking a single colony of each isolate on TZC (triphenyltetra zolium chloride) media and preserved for subsequent biochemical and molecular studies.

2.2 Biochemical Characterization of *R. solanacearum*

Potassium Hydroxide solubility test was performed for all the isolates. A loop full of each bacterium was placed on a glass slide in a drop of 3 % (v/v) KOH solution, stirred for 10 seconds and observed for the formation of slime threads and confirm the isolate as gram-negative bacteria.

2.3 Determination of Pathogenicity

To confirm the isolates of *R. solanacearum*, the pathogenicity test was performed in one month old seedlings by stem/soil inoculation method. A single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the centre of the TZC medium was selected for each group of isolates for pathogenicity test. At 30-40 days age of tobacco plants, bacterial suspension (approximately 10^8 CFU/ml) of each isolate representing a group will be injected in the leaves.

Tomato [Variety Goraka Thakkali], Brinjal [Variety Thinnaweli purple], Chilli [Variety CA-8] and Capsicum [Variety Hungeriun Yellow Wax] were inoculated with different isolates and observed for the wilting symptoms for up to one month of period. One pot contained two plants where one inoculum was inoculated to two spots of the two plants, to the lower part of the stem and upper part of the stem wrapping the inoculated area with wetted cotton.

2.4 Molecular confirmation of *Ralstonia solanacearum*

Bacterial DNA extraction method was utilized to extract DNA from the isolates. A loop full of bacteria cell culture was transformed into 9ml of Sucrose Peptone (SP) broth and bacteria was allowed to grow overnight with shaking at 150 rpm in the mechanical shaker. Bacterial DNA was extracted from 12 bacterial isolates based on cell lysis DNA extraction method (Weller *et al.*, 2000). The quality of the DNA of bacterial isolates verified after electrophorized in an 1% agarose gel electrophoresis.

2.5 PCR detection and identification of *Ralstonia solanacearum*

The 16S rRNA gene was amplified by PCR using *Ralstonia solanacearum* species specific primers RALS-F (5'-GAACGCCAACGGTGCGAACT-3') and RALS-R (5'-GGCGGCCTTCAGGGAGGTC-3') which amplify a fragment of 400 bp size (Weller et al., 2000). The reaction mixture contained a total volume of 10 µl. It contained 5 µl of PCR master mixture (25 units Taq DNA polymerase, 200 µm of each dNTP and 1x PCR buffer and 1.5mm $MgCl_2$), 0.8 µl of each primer (10mm), 0.5 µl of diluted (1:10) DNA template and 2.9 µl of sterilized distilled water.

PCR amplification was done with an initial denaturation at 94^0C for 5 minutes followed by 35 cycles of 1 minutes denaturation at 94^0C, 30 second annealing at 64^0C and 1 minute extension at 72^0C and final extension step was 10 minutes at 72^0C using a thermo cycler.

The PCR products obtained by amplification were electrophoresed on a 1.4% (w/v) agarose gel and visualized with a gel documentation system 1kb ladder was used as a marker.

2.6 Determination of Biovars through biochemical test

The isolates of *R. solanacearum* were differentiated into biovars based on their ability to utilize selected disaccharides (lactose, maltose and cellobiose) and hexose alcohols (mannitol, sorbitol and dulcitol) according to Hayward (1994).

The micro titer plates were incubated at 30ºC and examined at 3, 7 and 14 days for change of pH as described by Hayward in 1964 (Change of pH was indicated by the change of colour of reaction mixtures in wells, from green to yellow). Each test was replicated three times.

2.7 Race Identification based on host, pathogen interaction of Tobacco and Capsicum

The race identification was determined based on hypersensitive reaction (HR) on tobacco and capsicum plants using the leaf infiltration technique of Lozano and Sequeira (1970).

Table 1. Tobacco plant reaction to different races of *Ralstonia solanacearum* according to Lozanom and Sequeira (1970)

Race	Time period	Reaction type
1	24h	No visible symptoms
	36h	Dark brown lesion surrounded by a yellow zone
	8 days	Leaf wilting and yellowing
2	10 – 12h	Hypersensitive Reaction, infiltrated tissue glassy tissue thin, transparent, white necrosis
3	48h	Infiltrated tissue become yellow

2.8 Hypersensitive reaction of bitter gourd varieties against different isolates of *Ralstonia solanacearum*

Interaction between bitter gourd varieties (Maya, Matale Green-MG, TIA x MG and MG x IF6) and different *Ralstonia solanacearum* isolates was determined based on hypersensitive reaction (HR) using the leaf infiltration technique of Lozano and Sequeira (1970). The bacterial suspensions (OD 0.3 at 600nm) of all isolates of bitter gourd after 48h incubation were filtered into the interveinal areas on the lower side of fully expanded bitter gourd leaves using a disposable syringe with its needle removed and the pressure was gently applied and distilled water was used as control. Leaf reactions were recorded from 12, 24, 48 and 72 h up to 10 days for symptoms development in inoculated areas according to the Table 1.

3. Results and Discussion

3.1 Identification of bacterial wilt pathogen

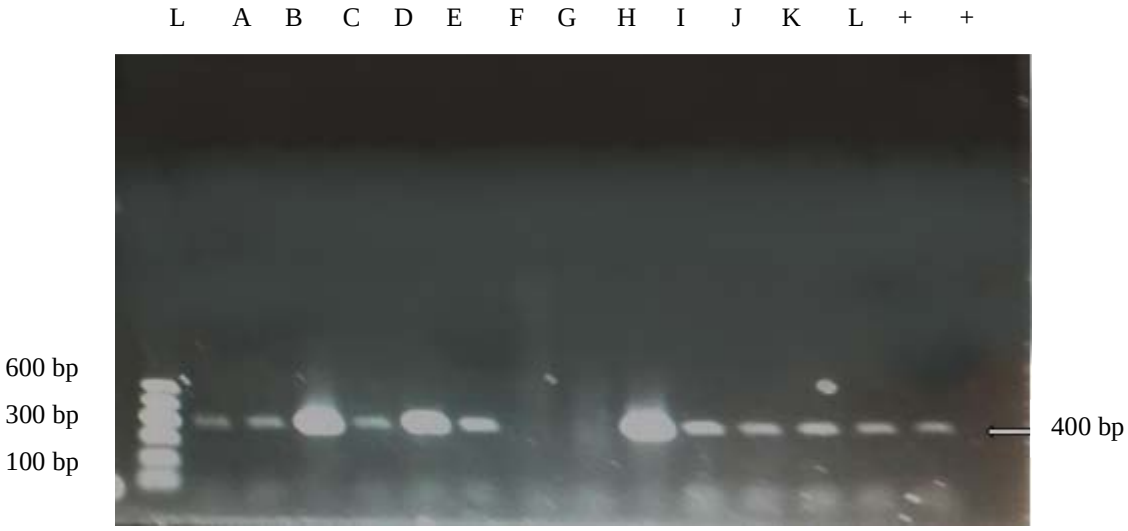
3% Potassium Hydroxide solubility test (3% KOH) was performed and formation of slime threads confirmed the isolates as gram negative bacteria. A single colony of isolates on TZC media showed virulent, fluidal, irregular and creamy white with pink at the centre. Based on KOH test and colony morphology, above isolates can be suspected as *Ralstonia solanacearum.*

3.2 Molecular identification of *Ralstonia solanacearum*

Molecular identification was carried out using specific primer pairs (RALS-F and RALS-R) which facilitated the amplification of an 400 bp amplicon.

The twelve isolates obtained from wilt infected bitter gourd gave PCR products approximately 400 bp with specific primers confirming that the all twelve isolates belongs to *Ralstonia solanacearum.*

***Ralstonia solanacearum* infected in Bitter gourd**



L - 100 kb ladder

	Location	Sampling	Biovar Reaction
A-	Mawanella	stem	biovar V
B-	Siyabalagaspitiya	Stem	biovar III
C-	Rambukkana-	Petiole	biovar III
D-	Siyabalagaspitiya	root	biovar V
E-	Siyabalagaspitiya	root	biovar III
F-	Mawanella	root	undetectable
G-	Mawanella	root	undetectable
H-	Rambukkana	root	biovarIII
I-	Mawanella	stem	biovar V
J-	Mawanella	leaf	biovar III
K-	Mawanella	leaf	undetectable
L-	Mawanella	root	biovar V
+	Positive samples		

3.3 Determination of Biovars through Biochemical test

Biochemical test of all 12 isolates from oxidized disaccharides (maltose, lactose and cellobiose) and alcohols by changing colour of the medium from green to yellow indicating that all the isolates are belongs to biovar III and V.

3.4 Race determination through the Tobacco and capsicum plant reaction

Tobacco and Capsicum plant reaction to different races of *Ralstonia solanacearum* according to Lozanom and Sequeira (1970), infiltrated tissue become yellow within 48h. So, we determined the all bacterial isolates are belongs to race 3.

Table 2. Biovar characterization and Race determination of *Ralstonia solanacearum*

Bacterial Isolates	Biovar Type	Race
A	V	3
B	III	3
C	III	3
D	V	3
E	III	3

F	Undetectable	3
G	Undetectable	3
H	III	3
I	V	3
J	III	3
K	Undetectable	3
L	V	3

3.5 Interaction between bitter gourd varieties and *Ralstonia solanacearum* isolates

The results of the infiltration of bitter gourd leaves with different 7 isolates of bacterial wilt induced only a chlorotic reaction of the infiltrated area that appeared 24–72 hours after infiltration. Bitter gourd leaves infiltrated with sterile water showed no reaction. Lozano and Sequeira, 1970 described that race III isolates of *Ralstonia solanacearum* caused only a discoloration of the infiltrated area. This experiment was repeated in two times.

Table 3. Variety, Isolate Interaction based on Hypersensitive Reaction

Ralstonia solanacearum Isolates	Bitter gourd Varieties			
	TIA x MG	Matale Green	Maya	MG x IF6
A	+ + +	+	+ + +	+ +
B	+	+	+ + +	+ + +
E	+ + +	+ + +	+ + +	+
F	+	+ + +	+ + +	+
G	+ +	+ + +	+ + +	+
J	+ +	+ + +	+ + +	+ + +
L	+ +	+	+ + +	+ + +

+ + + Susceptible – After 24 hours appeared light yellow colour lesion and after 72 hours remained it same colour.
+ + Moderately susceptible - After 24 hours appeared light yellow colour lesion and after 72 hours change into yellow colour.
+ Moderately Resistant - After 24 hours appeared yellow colour lesion and after 72 hours remained it same colour.

Variety Matale Green is generally known on moderately resistant for bacterial wilt and variety Maya is susceptible for bacterial wilt under field condition. According to the Variety, Isolate Interaction based on Hypersensitive Reaction, most popular bitter gourd variety of Maya was highly susceptible for the all isolates of *Ralstonia solanacearum*. Variety of Matale Green (MG) was susceptible with some isolates and moderately resistant with some isolates. Variety TIA x MG and MG x IF6 show variation based on isolates. Results numerated that there was a variation of pathogenicity of *Ralstonia solanacearum* isolates on different bitter gourd varieties.

4. Conclusion

Results numerated and identified the wilt pathogen of bitter gourd was *Ralstonia solanacearum.* All isolates from mid country areas of Sri Lanka belonged to Race 3 and Biovar V and Biovar III. There was an interaction between bitter gourd varieties and *Ralstonia solanacearum* isolates. The findings are relevant while devising a more targeted management approach to bacterial wilt of bitter gourd in mid country areas of Sri Lanka.

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