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Identification of the causal pathogen of bacterial wilt disease in cucurbitaceous crops and bean and its alternative weed hosts in Sri Lanka

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Abstract

Bacterial wilt caused by *Ralstonia solanacearum* is a destructive disease and one of the major constraints in vegetable production of Sri Lanka. Outbreak of bacterial wilt disease occurred in recent years for the first time in cucurbits and bean cultivated fields with 50-100% disease incidence. Hence, the present study was carried out with the objectives of isolation and molecular identification of causal pathogen of bacterial wilt of cucurbits and beans and to identify the weed hosts that harbour the bacterial wilt pathogen. Based on morphological and PCR analysis, gene sequence and homology search results, the causal pathogen of bacterial wilt disease in *Momordica charantia, Cucumis sativus, Luffa acutangula, Cucurbita maxima, Cucumis anguria, Citrullus lanatus* and *Phaseolus vulgaris* in Sri Lanka were identified as *R. solanacearum* with 99.37-100% similarity with Gene bank Accession CP022795.1, CP025986.1 and MF373815.1 of *R. solanacearum*. Twenty five out of 28 weeds species collected from Bacterial wilt infected fields were identified as symptomless carriers of *R. solanacearum*. All eight isolates from cucurbits and bean and 25 weeds species belong to biovar 3 and race 3 of *R. solanacearum*. This is the first report of *R. solanacearum* causing bacterial wilt in cucurbits, bean and weeds in Sri Lanka.

Key words: Bacterial wilt, Bean, Cucurbits, Molecular Identification, Weeds

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* has become an emerging disease and it has been ranked as the second most important bacterial pathogen causing 30- 90% yield losses in tomato and potato (Yuliar *et al*., 2015). *R. solanacearum* is a soil-borne bacterium that infects over 450 host species belonged to 54 plant families.

The crops such as potato, tomato, brinjal and pepper from family solanaceae are more susceptible to *R. solanacearum* (Wicker *et al*., 2007).

In Sri Lanka, bacterial wilt disease is widespread and a major constraint in the cultivation of solanaceous vegetables. Apart from solanaceous crops, previously reported non-host crops of *R. solanacearum* have become susceptible to bacterial wilt in the recent years. In 2017, outbreaks of a bacterial wilt disease of *Luffa acutangula* (ridge gourd), *Momordica charantia* (bitter gourd), *Cucumis sativus* (cucumber), *Cucumis anguria* (gherkin), *Cucurbita maxima* (pumpkin), *Citrullus lanatus* (water melon) and *Phaseolus vulgaris* (pole bean) occurred for the first time in the fields of cucurbits with 50-100% disease incidence at Bakamoona, Wariyapola, Wawela, Madamahanuwara, Gannoruwa and Pala kudawa subsequently to the first notice of this disease in bitter gourd fields at Marassana in 2016. Later, it was reported in bean cultivation at Muruthalawa in 2018. The apparent spread and destructiveness of the disease, which has increased over the years may be due to continues cultivation of susceptible crops in wilt -infected fields and changes in climatic conditions.

Knowledge on pathogen survival based on infection of alternative hosts and persistence of the pathogen in naturally infested soil with the hosts is fundamental to the development of control strategies. Therefore, further investigations are needed to determine whether weed and other crop species those are commonly found farmer fields could act as alternative hosts of *R. solanacearm* and it's biovars and races of the pathogen.

The objectives of the present study were isolation and molecular identification of causal pathogen of bacterial wilt of cucurbits (bitter gourd, cucumber, gherkin, pumpkin, ridged gourd and water melon) and beans and identify the weed hosts that harbor the bacterial wilt pathogen.

Materials and methods

Collection of wilt affected plants and asymptomatic weeds

Infected host plants *viz*. cucumber, bitter gourd, pumpkin, gherkin, ridge gourd, water melon and pole bean with typical symptoms of bacterial wilt were collected from different parts of the country during *Maha* 2017/18 and *Yala* 2018 seasons. The disease infected plants were diagnosed initially with visual symptoms and using the ooze test

(Hayward, 1994). Twenty eight weed samples with no appearance of disease symptoms were collected from bacterial wilt infected tomato and bean fields at Gannoruwa and Muruthalawa during *Yala* 2018 and *Maha* 2018/2019 seasons and subjected to ooze test.

Bacterial isolation from wilt affected plants and asymptomatic weeds

The wilted plant samples and weeds were washed under running tap water to remove sand and soil. The stem cuts were surface sterilized with 70% alcohol and the cut ends of each wilted sample and asymptomatic weeds were separately dipped into sterile water in test tubes for bacterial ooze. The bacterial suspension was then streaked on Sucrose Peptone Agar (SPA) medium and incubated at 28 ^{o}C for 24 hours for the growth of the bacterium. All the bacterial isolates were purified by streaking a single colony of each isolate on Tetrazolium Chloride (TZC) medium and preserved for subsequent biochemical and molecular studies. Extraction of *Ralstonia* bacteria from each weed samples was done as described by Pradhanang *et al*. (2000). Basal portion of the stem (1 cm) of each weed was macerated in plastic bags with 1 m*l* sterile Phosphate Buffer (PB). After 30 minutes, one loopful of each suspension was streaked on TZC medium.

Determination of biovars through biochemical test

The biochemical test for biovar characterization was carried out for seven cucurbits and bean isolates of *R. solanacearum* and 25 weed isolates of *R. solanacearum* based on the utilization/oxidation of selected disaccharides (lactose, maltose and cellobiose) and hexose alcohols (mannitol, sorbitol and dulcitol) as described by Hayward (1994). All of the inoculated tubes were incubated at room temperature (28 ^{o}C ± 2) and examined up to 30 days for colour change in the Hayward's medium from green to yellow. The changing of the colour was recorded as positive and biovars were determined based on that. Biovar 3 oxidizes both disaccharides and hexose alcohols whereas Biovar 1 does not oxidize neither hexose alcohols nor disaccharides, biovar 2 oxidizes only disaccharides, biovar 4 oxidizes only alcohols and biovar 5 oxidized all disaccharide and one hexose alcohol (Manitol) as described by Hayward (1994). Each test was repeated three times.

Hypersensitive Reaction (HR)

Hypersensitivity Reaction (HR) on Tobacco (*Nicotiana tabacum* L 'white burley') leaves by leaf infiltration technique described by Lozano and Sequeira (1970) was used for this. The bacterial suspensions (OD 0.3 at 600 nm) of virulent cultures of

33 isolates of cucurbits, bean and weeds after 48 hours of incubation were infiltrated into the interveinal areas on the abaxial surface of both tobacco and capsicum leaves using a needle removed disposable syringe. A little pressure was gently applied to the syringe to release the bacterial suspension and distilled water was used for the control. The reactions of test plants were then examined according to Lozanom and Sequeira (1970) at 12 h, 24 h, 48 h and 72 h up to 10 days for symptoms development in inoculated areas (Table 1).

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

Race		Reaction type
1	24 h	no visible symptoms
	36 h	dark brown lesion surrounded by a yellow zone
	8 days	leaf wilting and yellowing
2	10-12 h	HR reaction, infiltrated tissue glassy
		tissue thin, transparent, white necrosis
3	48 h	infiltrated tissue become yellow

Molecular confirmation of *R. solanacearum*

Bacterial DNA was extracted from 33 bacterial isolates (from 8 crops and 25 weed hosts) based on the cell lysis DNA extraction method described by Weller *et al*. (2000). The quality of the DNA of bacterial isolates was verified on 1.4% agarose gel electrophoresis.

PCR detection and identification of *R. solanacearum*

The 16S rRNA gene was amplified by PCR using *R. solanacearum* species specific primers RALS-F (5'-GAACGCCAACGGTGCGAACT-3') and RALS-R (5'-GGCGGCCTTCAGGGAGGTC-3'), which amplify a fragment of 400 bp (Weller *et al*., 2000). The reaction mixture contained a total volume of 10 µl of PCR master mixture (Promega, USA), 0.8 µl of each primer (10 mM), 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 °C for 5 min followed by 35 cycles of 1 min denaturation at 94 °C, 30 seconds annealing at 64 °C and 1 min extension at 72 °C and final extension step was 10 min at 72 °C using a thermo cycler (Labnet Gradient, Labnet Inc. USA). The PCR products obtained by amplification were analyzed on a 1.4 % (w/v) agarose gel and

visualized with a gel documentation system (ENDURO™ GDS,USA). Promega- G571A 1 kb ladder was used as a marker.

DNA sequencing and homology search

The eight PCR products (bacterial isolates of six cucurbits, one bean and one weed) were sent to Department of Molecular Biology and Biotechnology, Science Faculty, University of Peradeniya, Sri Lanka for sequencing. BLAST searches were performed for sequences obtained to find the similarity with sequence data in Gene Bank (http: www.ncbi.nlm.gov/ BLAST/).

Results and discussion

Field symptoms of affected crops

Disease symptoms of all cucurbits crops appeared in all the growth stages as patches or scattered individual plants with sudden drooping of leaves. The whole plant finally wilted, leaves became brown, dried and longitudinal lower stem sections showed a brown discoloration in the vascular tissues and disease incidence ranged between 20-70% in affected fields of cucurbits. Similar symptoms were initially appeared in beans during the vegetative stage and continued till fruiting stage and the disease incidence was up to 100 %. Bacterial ooze from the cut surface of the affected plants of cucurbits and bean was visible when dipped the cut end in water. None of the stem section sampled from non-wilted weed plants showed bacterial oozing when suspended in water.

Bacterial isolation from wilt affected plants and asymptomatic weeds

Eight bacterial isolates (Table 3) taken from cucurbits and beans produced typical *R. solanacearum*-type colonies that were smooth, irregular, round, creamy white fluidal colonies with pinkish center on TZC medium plates after 48 hours of incubation at 28±2 °C, proof of the presence of pathogenic bacteria as suggested by Klement (1990). Similarly, 25 bacterial isolates out of 28 weed samples (Table 2) also produced typical colony characters *R. solanacearum* on TZC medium. It indicated that *R. solanacearum* can survive on weed hosts without causing any wilt symptom. The colony characteristics of these isolates were consistent with previous descriptions of *R. solanacearum* (She *et al*., 2012).

Table 2. *R. solanacearum* (Rs) infected weeds collected from Bacterial wilt infected fields at Gannoruwa and Muruthalawa

Isolate code	Family	Botanical name	Common name	Rs on TZC
Rs-w1	Euphorbiaceae	*Euphobia thymifolia*	Kapumkeeriya	+
Rs-w2	Amaranthaceae	*Amaranthus viridis*	Kura thampala	-
Rs-w3	Compositae	*Vernonia cinerea*	Monarakudumbiya	+
Rs-w4	Poaceae	*Fimbristylis spp*	Maruk	+
Rs-w5	Fabaceae	*Mimosa pudica*	Nidikumba	-
Rs-w6	Verbenaceae	*Portulaca oleracea*	Gendapala	+
Rs-w7	Clemaceae	*Cleome viscosa*	Wal-abba	+
Rs-w8	Euphorbiaceae	*Phillanthus debilis*	Pitawakka	+
Rs-w 9	Cyperaceae	*Cyperus iria*	Thunessa	+
Rs-w10	Asteraceae	*Spilanthes paniculata*	-	+
Rs-w11	Asteraceae	*Ageratum conyzoids*	Hulanthala	+
Rs-w12	Euphobiaceae	*Euphobia hetaropilla*	Wal rubber	+
Rs-w13	Cyperaceae	*Cyperus rotundus*	Kaladuru	+
Rs-w14	Poaceae	*Eleusine indica*	Belathana	+
Rs-w15	Commelinaceae	*Commelina bengalensis*	Maha girapala	+
Rs-w16	Asteraceae	*Emilia sonchifolia*	Kadupahara	+
Rs-w17	Poaceae	*Chrysopogona ciculatus*	Thuththiri	+
Rs-w18	Poaceae	*Isachne globosa*	batadella	+
Rs-w19	Poaceae	*Pennisutum polystachion*	Rilathana	+
Rs-w20	Onagraceae	*Ludwigia octovalvis*	Walkarambu	+
Rs-w21	Lamiaceae	*Leucas zelanika*	Thumba	-
Rs-w22	Malvaceae	*Sida rhombifolia*	Babila	+
Rs-w23	Oxaliaceae	*Oxalis barrelieri*	Gas Abiliya	+
Rs-w24	Poaceae	*Paspalum distichum*	Diya thana	+
Rs-w25	Commelinaceae	*Commelina diffusa*	Heen girapala	+
Rs-w26		Unknown		+
Rs-w27		Unknown		+
Rs-w28		Un known		+

Rs on TZC = Ralstonia solnacearum growth on Tetrazolium medium

In Sri Lanka, weed species *Cyperus rotundus, Ageratum conyzoides, Euphorbia hirta* and *Ludwigia octovalvis* are more prevalent in vegetable growing areas both high

and low lands and were identified as symptomless carriers of *R. solanacearum* in this study. Similar studies reported by Lin *et al*. (1999) showed that nine species including *Cyperus rotundus, Ageratum conyzoides, Euphorbia hirta* and *Ludwigia octovalvis* were identified as symptomless carries of *R. solanacearum* belonged to race 1 and biovar 3 in Taiwan. Titatarn (1986) reported that the major weed species belonging to Asteraceae and *Ageratum conyzoides* and *Eupatorium odoratum* are very common and *R. solanacearum* prevalent in Thailand were biovars 3 and 4. Pradhanang *et al*. (2000) indicated that the role of non-solanaceous summer weeds for the persistence of biovar 2 of *R. solanacearum* in potato growing areas in the high hills of Nepal.

Biochemical test

Biochemical test of all 33 *Ralstonia* isolates oxidized all disaccharides (maltose, lactose and cellobiose) and alcohols (mannitol, sorbitol and dulcitol) by changing colour of the medium from green to yellow within 14 days, indicating that all the isolates of *R. solanacearum* from 25 weed hosts (Table 2), 7 cucurbits and 1 bean isolates (Table 3) belong to biovar 3. It implies that biovar 3 was the most dominating one in sample collected areas (Table 3). Wicker *et al*. (1994) reported an outbreak of bacterial wilt on cantaloupe, zucchini, pumpkin and cucumber, which was caused by *R. solanacearum* biovar 1 in French West Indies.

Table 3. Biovar characteristics of *R. solanacearum* isolates from cucurbits and bean in Sri Lanka

Isolate No.	Isolated Host	Location	Rs on TZC medium	Biovar
Rs-cu1	Cucumber	Bakamoona	+	III
Rs-cu2	Cucumber	Wariyapola	+	III
Rs-rg	Ridge gourd	Wawela	+	III
Rs-bg	Bitter gourd	Gannoruwa	+	III
Rs-gh	Gherkin	Bakamoona	+	III
Rs-pk	Pumpkin	Madamahanuwara	+	III
Rs-wm	Water melon	Palavi kuda	+	III
Rs-bn	Bean	Muruthalawa	+	III

Rs on TZC = Ralstonia solnacearum growth on Tetrazolium medium

Hypersensitive reaction (HR)

The results of the infiltration of tobacco leaves with seven isolates of cucurbits, one isolate of bean and 25 isolates of weeds induced yellowing of the infiltrated area (Table 1), which appeared 48-72 hrs after the infiltration. Tobacco leaves infiltrated with sterile water showed no reaction (Figure 1). Lozano and Sequeira (1970) described that race 3 isolate of *R. solanacearum* caused only a discoloration of the infiltrated area. All the isolates of cucurbits, bean and asymptomatic weeds (collected from bacterial wilt infected fields) tested in this study belonged to race 3. Mathew *et al*. (1994) reported that bacterial wilt of pumpkin and snake gourd, bitter gourd and ridge gourd in Kerala, India caused by *R. solanacearum* race 1 and biovar 3.

Figure 1. HR reaction on Tobacco leaves 48 hr after infiltration with *R. solanacearum* isolates.

Molecular identification of Ralstonia solanacearum

Molecular identification was carried out using specific primer pairs (RALS-F and RALS-R), which amplified approximately 400 bp portion. The eight isolates obtained from wilt infected pumpkin, ridge gourd, cucumber, bitter gourd, gherkin, water melon and bean gave PCR products (approximately 400bp) with species specific primers confirming that all the eight isolates belong to *R. solanacearum* (Figure 2).

The expected fragment size of 400 bp was also amplified with DNA of 25 bacterial isolates obtained from weed samples (Figure 3 and 4) with *R. solanacearum* specific primers of RALS-F and RALS-R. It confirms the identity of them as *R. solanacearum*. The results indicated that symptomless weeds that carried *R. solanacearum* occurred in infected fields act as the potential host of *Ralstonia solanacearum* in Sri Lanka.

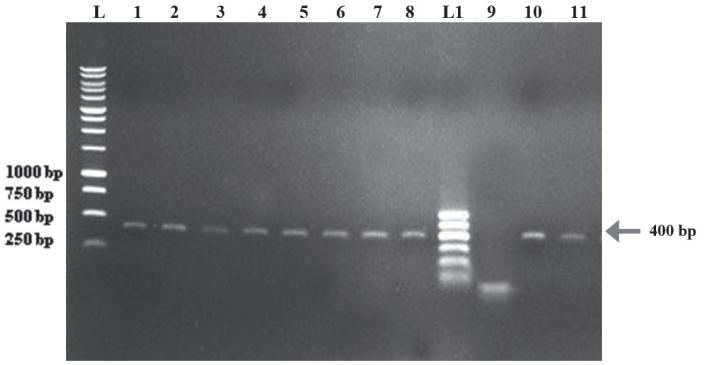

Figure 2. Identification of isolates of *R. solanacearum* in different crops by using specific primers. Lanes L: DNA ladder 1 kb, 1: Rs-cu1, 2: Rs-cu2, 3: Rs-rg, 4: Rs-bg, 5: Rs-gh, 6: Rs-pk, 7: Rs-wm, 8: Rs-bn, L1: Ladder 100 kb, 9: negative control, 10 & 11: Positive control (tomato & brinjal)

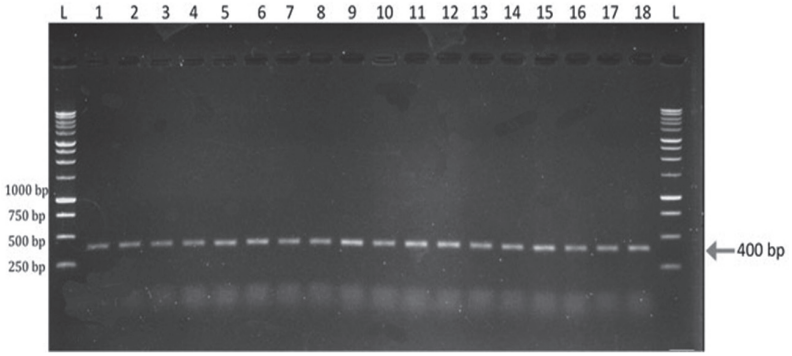

Figure 3. Identification of isolates of *R. solanacearum* in different weed samples by using specific primers. Lanes L: DNA ladder 1 kb, 1: Rs-w1, 2: Rs-w3, 3: Rs-w4, 4: Rs-w6, 5: Rs-w7, 6: Rs-w8, 7: Rs-w9, 8: Rs-w10, 9: Rs-w11, 10: Rs-w12, 11:Rs-w13, 12:Rs-w14, 13:Rs-w15, 14: Rs-w16, 15:Rs-w17, 16:Rs-w18, 17: Rs-w19, 18: Rs-w20

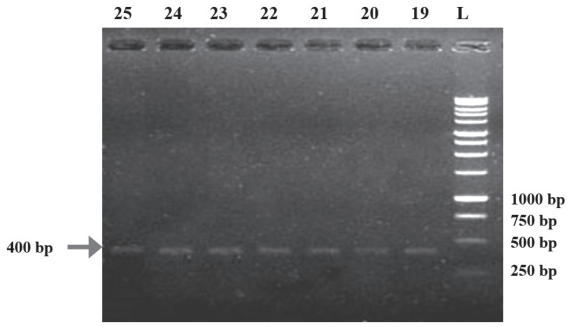

Figure 4. Identification of isolates of *R. solanacearum* in different weed samples by using specific primers. Lanes 19: Rs-w22, 20: Rs-w23, 21: Rs-w24, 22: Rs-w25, 23: Rs-w26, 24: Rs-w27, 25: Rs-w28, L: DNA ladder 1 kb

DNA sequencing and homology search

Results of the Homology search revealed that all the *R. solanacearum* isolates of cucurbits, bean and weeds in Sri Lanka were highly matched with *R. solanacearum* strain SL2330 (Accession No. CP022795.1- *Solanum tuberosum* /Korea), *R. solanacearum* strain TRsP (Accession No. MF373815.1- *Solanum lycopersicum*/ India) and *R. solanacearum* strain RSCM (Accession No. - CP025986.1 - *Cucurbita maxima*/China) with 99.32 -100 % similarity (Table 4). Similar studies conducted for molecular identification of causal pathogen of bacterial wilt disease of brinjal and tomato crops at Gannoruwa, Sri Lanka have given similar results with DNA sequencing data (Acc. No. CP022795.1, CP025986.1 and MF373815.1) showing 96.89 % similarity (unpublished data).

Table 4: Results of homology search of *R. solanacearum* isolates

Isolate No.*	Best homologue given by BLAST	Query cover (%)	E value	Identity (%)	Sequence ID of the best homologue
Rs-cu2, Rs-rg, Rs-gh, Rs-pk, Rs-wm & Rs-bn	*R. solanacearum* strain SL2330, TRsP & RSCM	100	4e-150	100	CP022795.1, CP025986.1 & MF373815.1
Rs-bg	*R. solanacearum* strain SL2330, TRsP & RSCM	100	8e-147	99.32	CP022795.1, CP025986.1 & MF373815.1
Rs-w11	*R. solanacearum* strain SL2330, TRsP & RSCM	100	3e-146	99.32	CP022795.1, CP025986.1 & MF373815.1

**Rs-cu2 –Cucumber, Rs-rg -Ridge gourd , Rs-gh- Gherkin, Rs-pk- Pumpkin, Rs-wm- Water melon, Rs-bn-Bean, Rs-bg- Bitter gourd and Rs-w11 -Ageratum conyzoids*

On the basis of biochemical and molecular identification and homology search all bacterial isolates obtained from cucurbits, bean and twenty five symptomless weeds in this study were identified as *R. solanacearum* race 3 and biovar 3. This is the first report of bacterial wilt of cucurbits, bean caused by *R. solanacearum* in Sri Lanka, which have been reported previously in cucumber, bitter gourd and Ridge gourd in some other Asian countries (Mathew *et al*., 1994; Mondal *et al*., 2016; She *et al*., 2017).

Controlling bacterial wilt is difficult because the pathogen has an extremely broad host range, and is able to survive in the soil in the absence of the host plant. Moreover, it can colonize in both host and non-host plants including many weeds,

without producing visible symptoms (Hayward, 1994). In Sri Lanka, it is observed that most of the solanaceous, cucurbits and bean cultivation fields were affected by bacterial wilt disease in recent years due to continuous cultivation of susceptible host plants with poor weeding throughout the cropping season. Therefore, weed control and crop rotation with non-host crops should be practiced, especially in bacterial wilt infected fields to eliminate an inoculum source for the *R. solanacearum* bacterium.

This finding contributes to update the existing information on *R. solanacearum* pathogen in Sri Lanka and useful in the development of molecular methods for strain identification and establishing new strategies for disease control.

Conclusion

Based on 16S rRNA gene sequence and homology search results, the causal pathogen of bacterial wilt occurred in *Cucumis sativus, Momordica charantia, Luffa acutangula, Cucumis anguria, Cucurbita maxima, Citrullus lanatus* and *Phaseolus vulgaris* in Sri Lanka was identified as *R. solanacearum* with 100% similarity with Genbank Accessions CP022795.1, CP025986.1 and MF373815.1. 25 out of 28 weeds identified as symptomless carriers of *R. solanacearum*. All isolates of *R. solanacearum* from bean, cucurbits and weeds were virulent and belongs to race 3 and biovar 3.

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