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Characterization of *Ralstonia solanacearum* causing bacterial wilt in ginger

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ABSTRACT: Bacterial wilt pathogen *Ralstonia solanacearum* isolated from ginger, tomato, *Chromolaena*, chilli and potato, characterized for biovar, pathogenicity, infectivity titer, and intrinsic antibiotic resistance. The isolates were also characterized on the basis of their membrane protein pattern and amenability of the isolates for serological detection (NCM-ELISA) using *R. solanacearum* specific antibodies. All the ginger isolates were highly fluidal with characteristic spiral pink center on 2,3,5-triphenyl tetrazolium chloride-amended medium. Among the isolates characterized nine belongs to biovar 3 while one from potato to biovar 2. Among the various isolates tested, only ginger ones except one from Assam induced cent percent wilting in ginger cv. *Himachal* within a week. Plants were wilted even at a concentration of 3.2 X 10^2 cfu ml^{-1} in stem inoculation while in soil inoculation it was 10^5 cfu ml^{-1}. All the isolates were resistant to antibiotics tetracycline, polymyxin B sulphate and chloramphenicol and isolates GRS Tms and its spontaneous mutant were resistant to Rifamycin. Isolates were detected with NCM-ELISA and biovars on the basis of membrane protein pattern on SDS-PAGE and biovar specific protein from *R. solanacearum* could be isolated.

Key words: *Ralstonia*, ginger, NCM-ELISA, membrane protein

Ginger (*Zingiber officinale* Rosc.) is an important spice crop that supports the livelihood of many farmers in Kerala, Karnataka, Himachal Pradesh, Meghalaya, Sikkim, West Bengal and other North Eastern states of India. Apart from being used as vegetable, ginger is grown for several value added product. Among the major production constraints bacterial wilt or '*Mahali*' or blast of ginger caused by *Ralstonia solanacearum* Yabuuchi (Smith) is very serious. The disease is endemic in majority of the ginger growing areas viz., Kerala, Sikkim and many other northeastern regions of the country causes yield loss up to 100 per cent under conducive conditions. (Dohroo 1991; Mathew *et al.,* 1979; Sarma *et al.,* 1978). The disease has also been reported from other parts of world viz., Queensland (Hayward *et al.,* 1967), Hawaii (Ishii and Aragaki, 1963), Mauritius and Malaya (Orian, 1953), Korea Republic (Choi and Han, 1990), China (Zheng -Xian Ming *et al.,* 1995) and Indonesia (Mulya *et al.,* 1990).

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Though the causal organism has been identified as *Ralstonia solanacearum* the characteristic features of *R. solanacearum* causing bacterial wilt in ginger are lacking in India. The present article deals with the pathogenic behavior, intrinsic antibiotic resistance, amenability of the isolates for serological detection and the strain differentiation by membrane protein pattern of *R. solanacearum* collected from different ginger growing areas of India and its relationship with strains of *R. solanacearum* isolated from tomato (*Lycopersicon esculantum),* chilli (*Capsicum annuum)*, potato (*Solanum tuberosum*) and eupatorium *(Chromolaena odorata*).

MATERIALS AND METHODS

Collection and phenotypic characterization of *Ralstonia solanacearum*

The pathogen *Ralstonia solanacearum* was isolated from wilted ginger plants collected from different locations of Kerala, Assam and West Bengal following standard procedure (Kelman, 1954; Mehan, 1995). *R. solanacearum* colonies appeared

after 36 hours of incubation at 28 °C as typical white fluidal with spiral pink center were purified. A loopful of bacterial growth was suspended in sterile distilled water and kept at 4 °C for short-term storage, while at –80 °C in 20 per cent glycerol for long-term storage.

For routine work media used was sucrose peptone agar (Mehan, 1995), CPG medium (Kelman, 1954), King's B medium (King *et al*., 1954) and semi selective medium (Hara and Ono, 1983). Catalase and salt tolerance tests were performed in nutrient broth. Biovar characterization of *R. solanacearum* was performed following the methods of Hayward (1960) with slight modification, i.e. use of microfuge tubes (1.0 ml) and microtiter plates (200 µl) along with conventional test tubes (3 ml) with bromothymol blue as pH indicator.

Variation in virulence of *R. solanacearum* and infectivity titer

Pathogenicity of *R. solanacearum* was tested on 45 days old ginger plants by stem inoculation method. One hundred microlitre of inoculum (10^8 cfu ml^{-1}) multiplied on CPG medium was placed on pinprick portion in between bottom leaf sheath and pseudostem near collar region and ten such inoculated plants were kept at 30°C. For infectivity titer assay, cells of *R. solanacearum* multiplied in sucrose peptone broth were washed free of medium (5000 rpm/20min), resuspended in sterile distilled water and 10-fold dilutions of cell suspension were made up to 10^{-9}. One hundred microlitres of diluted cell suspension from 10^{-8}, 10^{-6} and 10^{-4} was spread plated on sucrose peptone agar for estimation of colony forming units of *R. solanacearum* and another 100 µl was inoculated at base between last leaf sheath and pseudostem of 45 days old ginger plants. In the next experiment 1ml of bacterial suspension from different dilutions were poured around the base of the plant. The data on wilt incidence was recorded until the disease symptom appeared in all the inoculated plants.

Intrinsic antibiotic resistance of *Ralstonia solanacearum*

For antibiotic resistance Kanamycin (50 µg ml^{-1}), Nalidixic acid (40µgml^{-1}), Chloramphenicol (20 µg ml^{-1}), Streptomycin (100 µg ml^{-1}), Ampicillin (100 µgml^{-1}), Gentamycin (10µgml^{-1}), Tetracycline (15 µg ml^{-1}), Rifamycin (50 µg ml^{-1}) and Polymyxin B sulphate (10 µg ml^{-1}) were incorporated in sucrose peptone agar. A loopful of bacterial suspension was streaked on such amended medium, the plates were incubated at 30°C and growth was recorded after 48 h and compared with growth on unamended media

Serological diagnosis of *R. solanacearum*

Serological diagnostic kit (CIP–NCM-ELISA) obtained from International Potato Center, Lima, Peru was used to know the amenability of these isolates for serological detection. Using antibodies rose against potato isolates of *R. solanacearum*, ELISA was performed on nitrocellulose membrane as per the protocol of Priou *et al,* (1999).

Characterization of *R. solanacearum* based on membrane protein pattern

Isolation of membrane protein from ten isolates of *R. solanacearum* was carried out following the protocols suggested by Dianese and Dristig (1994). Isolates of *R. solanacearum* were grown overnight in medium 523 of Kado and Heskette (1970) at 30°C on a rotary shaker at 150 rpm. Late logarithmic phase cells were harvested by centrifugation at 13200g for 20min at 4°C and then washed twice in cold buffer (3.3mM Tris-Cl, pH 7.4). The cells were subjected to lysis in lysozyme (100 µg ml^{-1} of 10mM Tris-Cl + 0.75M sucrose, pH 7.4). After incubation for 10min in ice the products were homogenized in polytron homogenizer (Kinematica). The fraction containing total cell membrane was centrifuged at 36900g for 60 min to obtain membrane protein. The isolated protein was harvested in 50 µl buffer (3.3mM Tris-Cl, pH-7.4 + 0.25mM sucrose) and quantified (Bradford 1976). The isolated protein was separated in discontinuous PAGE system with a stacking gel containing acrylamide (5%) on top of a acrylamide analytical gel (12%) both containing SDS (0.1%).

RESULTS AND DISCUSSION

Fluidal colonies with characteristic pink centre appeared after 48 h on CPG medium. The colony morphology and key biochemical characters i.e. absence of growth on NaCl amended medium, catalase positive reaction, growth at 37°C, absence of fluorescence on Kings medium B and orange

fluorescence on Nile blue amended medium confirmed the identity of bacterium as *Ralstonia.* Further characterization revealed that isolate from ginger, *Chromolaena*, chilli and tomato belongs to *R. solanacearum* biovar 3, while potato to biovar 2 (Table 1). Earlier Hayward *et al,* (1967) identified biovar 3 and biovar 4 as wilt causing bacteria from ginger in Queensland.

Observations on colony morphology on different media revealed that the colonies were irregular, white and fluidal with incubation period of 48-72 hours on CPG and SMSA. Strains from ginger and other hosts could be differentiated on CPG medium on basis of colony fluidity. Colonies of ginger strains were highly fluidal with characteristic spiral pink centre whereas in the case of other strains fluidity and pink centre was less conspicuous. The colonies were irregular, highly fluidal, white and without pigmentation on King's B while on sucrose peptone agar colonies these were round to irregular,

Table 1. Details of *Ralstonia solanacearum* isolates obtained from ginger and other hosts

Isolate*	Crops & Location	Colony character on CPG medium	Pathogenicity to ginger	Biovar**
GRS Tms	Ginger, Thamarassery, Kerala	Incubation time: 36-72 h White, Fluidal, spiral pink centre, irregularity shaped.	Highly pathogenic	3
GRS Tms Rif^R	Spontaneous rifamycin resistant mutant of GRS Tms, Thamarassery, Kerala	Incubation time: 48-72 h White, Fluidal, spiral pink centre, irregularly shaped.	Highly pathogenic	3
GRS Pul	Ginger, Pulpally, Kerala	Incubation time: 48-72 h White, Fluidal, spiral pink centre, irregularly shaped.	Highly pathogenic	3
GRS Kki	Ginger, Kahikuchi, Assam	Incubation time: 48-72 h White, less fluidal, spiral pink centre, irregularly shaped	Non pathogenic	3
GRS Vyt	Ginger, Vythiri, Kerala	Incubation time: 48-72 h White, highly fluidal, spiral pink centre, irregularly shaped	Highly pathogenic	3
GRS Ktm	Ginger, Kothamangalam, Kerala	Incubation time: 48-72 h White, Fluidal, spiral pink centre, irregularly shaped	Weakly pathogenic	3
TRS Cal	Tomato, Calicut, Kerala	Incubation time: 48-72 h White, Highly fluidal, dark pink centre, irregularly shaped, rarely round.	Non pathogenic	3
ERS Cal	*Chromolaena*, Calicut, Kerala	Incubation time: 48-72 h White, Highly fluidal, dark pink centre, irregularly shaped, elongated colonies, rarely round.	Non pathogenic	3
PRS Pun	Potato, Pundibari, West Bengal	Incubation time: 48-72 h White, Highly fluidal, spiral pink centre, irregularly shaped, rarely round	Non pathogenic	2
CRS Avl	Chilli, Ambalavayal, Kerala	Incubation time: 48-72 h White, Highly fluidal, dark pink centre, irregularly shaped, rarely round.	Non pathogenic	3

*All isolates are resistant to Chloramphenicol (20 µg ml^{-1}), Tetracycline (15µg ml^{-1}) and Polymyxin (10µg ml^{-1})
** All isolates reacted strongly with Rs specific antibodies as observed in NCM-ELISA

Table 2. Infectivity titer of *Ralstonia solanacearum* in ginger

Inoculum concentration (cfu/ml)	Pseudostem inoculation		Soil inoculation	
	Initiation of disease (Days)	wilt (%)	Initiation of disease (Days)	wilt (%)
3.2 x 10^8	6	100(8)	12	100(19)
3.2 X 10^7	6	100(9)	12	30
3.2 X 10^6	7	100(20)	13	30
3.2 X 10^5	7	100(15)	14	10
3.2 X 10^4	8	80	*	*
3.2 X 10^3	8	80	*	*
3.2 X 10^2	9	80	*	*

Figures in parenthesis indicate number of days to wilt all the inoculated ginger plants.
*- No wilt incidence

creamy white and fluidal. All the pathogen strains were resistant to chloramphenicol (20 µg ml^{-1}), tetracycline (15 µg ml^{-1}) and polymyxin (10 µg ml^{-1}). Among the strains, GRS Tms and its spontaneous mutant could resist antibiotic rifamycin at 50 µg ml^{-1} (Table 1).

Pathogenicity of isolates

Among the different *Ralstonia solanacearum* strains tested, all the ginger ones except one from Assam induced wilt symptoms in ginger (Table 1). *Ralstonia solanacearum* from tomato, *Chromolaena*, chilli and potato were found non-pathogenic to ginger. It is interesting to note that the *R. solanacearum* from *Chromolaena*, a common weed of ginger fields was not pathogenic on ginger though it belonged to biovar 3. Wilting of plants started 6-7 days after inoculation and all the inoculated plants wilted with in 8-20 days (Table 2). Zehr (1970) has also found the wilting of ginger plants with in 10 days of inoculation with virulent isolates of *R. solanacearum*. However, Hayward *et al,* (1967) grouped the ginger isolates in to two groups i.e., the group 1, which is biovar 4, induced wilt in 14 and 21 days of stem and root inoculation while in group 2 (belong to biovar 3) plants wilted over a period of 6 weeks. But in the present investigation in India, biovar 3 was found quick wilting pathogen as it took only 6 days to initiate wilting.

Infectivity titer of *Ralstonia solanacearum* in ginger

Wilting started after 6 days of inoculation at the concentrations of 3.2 x 10^8 and 3.2 x 10^7 cfu ml^{-1} when the inoculum was placed in between bottom leaf sheath and pseudostem. At concentrations of 3 x 10^8 cfu ml^{-1} the wilting started after 6 days and complete wilting was observed on 8^{th} day (Table 2). However, in the case of inoculum poured around the base of the plant after pinprick, wilting was noticed after 12^{th} day and there was no wilting below the concentration of 3.2 x 10^5 cfu ml^{-1}.

Reactions of *R. solanacearum* with antibodies

NCM-ELISA originally developed for detection of *R. solanacearum* in potato seed tubers (Priou *et al.,* 1999) was adopted and compared with positive control strips provided in the kit (Fig. 1). All the

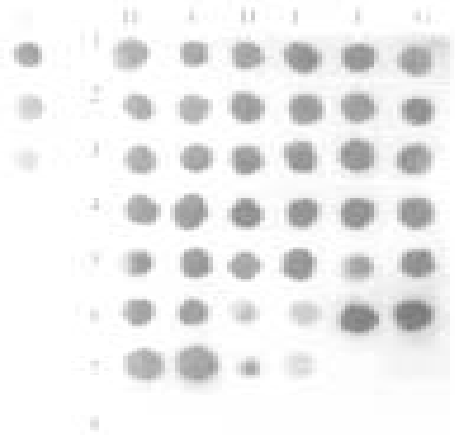

Fig. 1. NCM-ELISA of different isolates of *R. solanacearum.* Dark spot indicates positive serological reaction with Rs specific antibodies

Table 3. SDS-PAGE analysis of membrane protein of *R. solanacearum*

Isolate	Yield µg/ml	Rf value*	Molecular Weight (Kd)
Gin Tms/GRS Tms	27.45	0.54, 0.57, 0.73	42.7, 40.5, 26.8
GRS Tms Rif^R	27.30	0.54, 0.57, 0.73	42.7, 40.5, 26.8
GRS Pul	23.9	0.54, 0.56, 0.73	42.7, 41.2, 26.8
GRS Kki	35.4	0.52, 0.56, 0.73	45.4, 41.2, 27.4
GRS Vyt	32.2	0.54, 0.56, 0.72	43.5, 41.6, 27.3
GRS Ktm	32.1	0.54, 0.57, 0.72	42.7, 39.8, 26.3
TRS Cal	31.9	0.54, 0.57, 0.73	42.7, 39.8, 26.3
ERS Cal	27.4	0.53, 0.56, 0.72	44.9, 40.7, 26.5
PRS Pun	34.2	0.47, 0.56, 0.72	52.1, 41.5, 26.5
CRS Avl	23.9	0.54, 0.56, 0.72	43.6, 41.5, 26.5

ginger, tomato and *Chromolaena* strains gave positive colour reaction at a concentration of 10^8 cfu/ml, which broaden the scope of the kit for strains isolated from other hosts.

Membrane protein pattern of the pathogen

Ten isolates representing biovar 3 and biovar 2 were characterized using membrane protein pattern. (Table 3). The isolated membrane protein was separated in discontinuous PAGE system and the banding pattern was analyzed using Alpha imager gel documentation system. All the isolates were resolved in to two groups. Similarity index using unbiased Pair Group Method with Arithmetic Averages (UPGMA) was calculated and cluster analysis was performed using NTSyspc (Numerical Taxonomy and Multivariate Analysis System) software. All the isolates belonging to biovar 3 formed one cluster, while the biovar 2 from potato formed separate. Dristig and Dianese (1990) characterized *R. solanacearum* representing all the biovars based on their membrane protein pattern. A protein band with molecular weight 43-45 Kda could be found only in biovar 3 strains from ginger, tomato, *Chromolaena* and chilli (Fig 2). Earlier Dianese and Dristig (1994) found 39 Kda protein in all three biovars independent of their host and locality origin. The biovar specific protein could be exploited for raising specific antibodies for developing a diagnostic kit. The other two bands with molecular weights, 41 Kda and 27 Kda, could be species-specific proteins. A band with molecular weight of 51 Kda found only in biovar 2 which could be specific for this biovar.

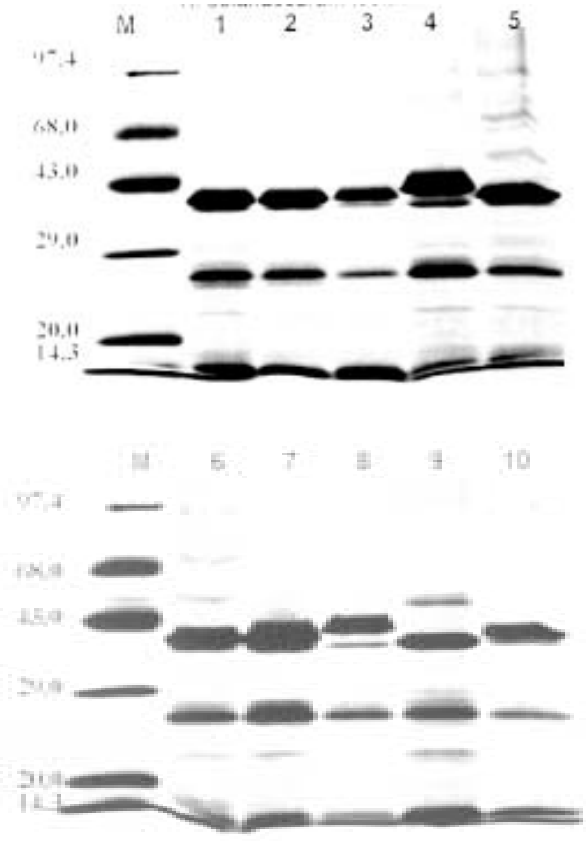

Fig. 2. Membrane protein pattern of *R. solanacearum* isolates. M- Protein marker (Kda), 1. GRS-Tms, 2. GRS Tms Rif^R 3. GRS-Pul, 4. GRS-Kki, 5. GRS-Vyt, 6. GRS-Ktm 7. TRS-Cal, 8. ERS-Cal 9. PRS Pun 10. CRS Avl
1-5: Biovar 3 from ginger, 6: Biovar 3 (ginger), 7: Biovar 3 (tomato), 8: Biovar 3 (Chromolaena), 9: Biovar 2 (potato), 10: Biovar 3 (capsicum)

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