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Small cardamom *(Elettaria cardamomum* Maton.) and ginger (*Zingiber officinale* Roxb) bacterial wilt is caused by same strain of *Ralstonia solanacearum*: a result revealed by multilocus sequence typing (MLST)

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Abstract Bacterial wilt in cardamom (*Elettaria cardamomum* Maton) was observed in Kerala state of India. Infected plants showed wilting wherein all leaves roll or curl upward towards the midrib centre, turn yellow, and the whole plant finally dies; the collar region shows water-soaked lesions initially and turns dark brown eventually; copious quantity of bacterial exudate is observed on the cut end of the pseudostem. The bacterium was identified as *Ralstonia solanacearum* based on a panel of phenotypic characters such as fluidal white colony on Kelman's medium, biovar assay and biolog assay (BiologGN), and genotypic characters such as Multiplex-PCR based phylotyping, sequences of *16S rDNA, 16-23S* intergenic region, and *rec*N gene. Collectively these tests revealed that the *R. solanacearum* infecting cardamom belong to biovar 3 and phylotype 1 confirming its Asian origin. Upon soil inoculation, the bacterium caused typical wilting of the cardamom plants in three weeks and ginger plantlets in two weeks. Cross transmissibility of the bacterium was observed in cardamom and ginger wherein the plants succumbed to wilt when *R. solanacearum* from either of the host was inoculated. BOX-PCR fingerprinting revealed that the strain is identical (100%) to a ginger strain of *R. solanacearum,* which is widely prevalent in the Indian subcontinent. Furthermore, Multilocus Sequence Typing (MLST) based strain comparison confirmed that cardamom and ginger strain were identical to each other at 11 loci. Apart from striking phenotypic and genotypic (allelic) similarities, geographical origin, and cross transmissibility of the cardamom strain of *R. solanacearum* strongly suggest that the new occurrence of wilt of cardamom in India could have an origin in bacterial wilt of ginger. Perusal of records on *Ralstonia*-induced bacterial wilt in crop plants, particularly among the Zingiberaceae family, reveals that this is a new report of bacterial wilt disease in small cardamom.

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Cardamom is one of the important spice crops cultivated in India, Guatemala, Tanzania, Sri Lanka, El Salvador, Vietnam, Laos and Cambodia. Cardamom plants expressing unusual wilt symptoms were observed in the Wayanad district of the state Kerala in South India in June 2010 (Fig. 1a). The symptoms are those of a typical vascular wilt wherein all the leaves of the plant rolled or curled inward (Fig. 1b) and turned golden yellow starting from the lower leaves; the collar region of the plants showed water soaked dark brown lesions (Fig. 1c). This kind of wilting symptoms had not been reported in small cardamom plantations under natural conditions. Copious quantities of bacterial exudate could be observed on the cut end of the stem and the same could stream out upon contact with water (Fig. 1d). The bacterial exudates obtained from the wilt-affected cardamom plant were characterized by a panel of biological, phenotypic and genotypic methods. The identity of the bacterium was further confirmed as *R. solanacearum* (99.9% probability) by a Biolog-based identification scheme (BiologGN plates). Hayward's (1964) biovar testing revealed that the *R. solanacearum* isolated from cardamom belong to Biovar 3. The multiplex PCR-based phylotyping suggested by Fegan and Prior (2005) and Opina et al. (1997) revealed that the cardamom strain of *R. solanacearum* and the ginger strain belong to phylotype 1 confirming their Asian origin (data not shown). The identity of strain was further confirmed by

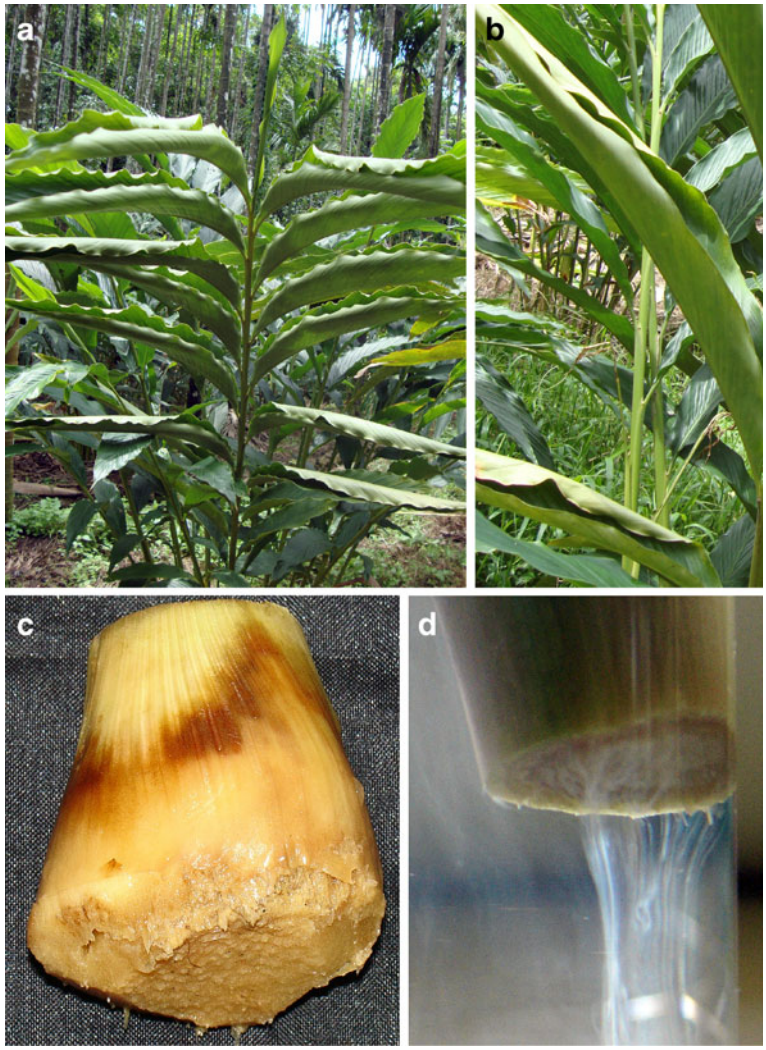

Fig. 1 **a** Natural occurrence of bacterial wilt in 4 years old cardamom plants. **b** Typical upward curling of leaves in the wilted plants. **c** Dark brown lesion on the base of the plants. **d** Bacterial streaming from the cut end of stem

comparing the sequences of rRNA (Taghavi et al. 1996; Poussier et al. 2000) and *rec*N gene. 1,465 bp of 16S rDNA (**GenBank Accession: JF523189**), 1,303 bp of 16-23S rDNA (**GenBank Accession: JF523188**) and 1,169 bp of *rec*N *(***GenBank Accession: JF523198**) were used for blast analysis (Altschul et al. 1997). The strain from cardamom displayed 100% similarity for 16S rDNA with the fully sequenced genome of *R. solanacearum* GMI1000 (Biovar 3 strain from tomato), and 99% similarity with five other strains whose full genome is published. For 16-23S and *rec*N sequences, the strains displayed 99% identity with the fully sequenced genome of GMI1000. For *rec*N based identification, primers (*Rec*N3F: gatttcgtcatcgtccatgc, *Rec*N5R: atcaccgatcgctaggc) were designed from the genome of *R. solanacearum* GMI1000 by Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and validated *in silico* in oligocalculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) prior to use.

The reaction mixture (50 μl) contained template DNA 100 ng; 1× PCR buffer; $MgCl_2$ 1.5 mM; each dNTP 200 μM; DMSO 6%; primers 10 pmoles and *Taq* DNA polymerase 1 unit. PCR was preceded by a denaturation at 96°C/9 min, followed 30 cycles at 95°C/1 min, annealing temperature at 60°C/1 min and extension temperature of 72°C/2 min with a final extension step of 72°C/10 min. PCR amplicon was purified and sequenced from both ends. Sequences were assembled using Vector NTi (Invitrogen) software package and were subjected to analysis.

Pathogenicity of isolates was determined on 4-month-old cardamom plantlets (Cultivar: *IISR-Suvasini*) and ginger plantlets (Cultivar: *Himachal*) by soil inoculation method by pouring 100 ml of *R. solanacearum* suspension (~1×10^8 CFU/ml sterile deionized water) around the collar region of each transplant. The cardamom plantlets showed wilting of plants 3 weeks post inoculation wherein leaves showed upward curling toward the midrib centre (Fig. 2), turned yellow, and the plant eventually died. The strain obtained from cardamom could wilt cardamom plantlets in 21–24 days and ginger plants in 10–12 days post inoculation. Strains of *R. solanacearum* that cause rapid wilting in ginger has been reported in India (Kumar and Sarma 2004, Kumar et al. 2004). Interestingly, cardamom plantlets succumbed to wilt three weeks post inoculation with the *R. solanacearum* strain isolated from naturally infected ginger (GRs-Tms) indicating functional similarity of the strains obtained from either host. However, upward curling of leaf margins in wilted cardamom plantlets and the slow spread in wild were opposite to the symptoms of ginger bacterial wilt which showed downward curling of leaves and spread fast in the field, often termed "ginger blast".

Fig. 2 Typical upward curling of leaves upon pathogen inoculation

R. solanacearum isolated from cardamom was compared with three other strains causing bacterial wilt of ginger, paprika (CRs-Per) and potato (PRs-Pun) by BOX-PCR *(*Rademaker et al. 1997) and Multilocus Sequence Typing (MLST) based on conserved gene sequences. Box-PCR profiles were converted into a binary matrix of presence/absence of each amplicon assuming that the amplicons with identical molecular size in different individuals were homologous. The data was used to construct a Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram using FreeTree software (Pavlicek et al. 1999) and a similarity coefficient was calculated (Table 1). *R. solanacearum* from cardamom shared all the BOX-PCR amplicons with the ginger strain indicating the close similarity among *R. solanacearum* infecting

Table 1 Similarity matrix between the isolates of *R. solanacearum* isolated from Cardamom and other strains based on BOX-PCR DNA profiling

	PRs-Pun	CRs-Per	GRs-Tms	CaRs-Mep
Potato: PRs-Pun		0.38095	0.57143	0.57143
Paprika: CRs-Per	0.38095		0.84615	0.84615
Ginger: GRs-Tms	0.57143	0.84615		1.00000
Cardamom: CaRs-Mep	0.57143	0.84615	1.00000	

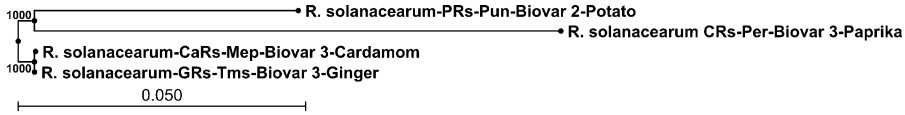

Fig. 3 Tree plot constructed by NJ method using 5,372 bp subjected to 1,000 bootstrap trials showing the relationship of *Ralstonia solanacearum* isolated from cardamom with other isolates. Strains representing potato (Biovar 2) and paprika (Biovar 3) served as out-groups. Bootstrap values are shown at the nodes

Zingiberaceae family members. We have further genotyped the pathogen by MLST, which exploits sequence data of *pps*A, *gap*A, *gdh*A, *adk*, *gyr*B, *fli*C, *hrp*B, and *egl* loci (Castillo and Greenberg 2007). A database of alleles (http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl) for these loci has been published (Almeida et al. 2010) which was exploited to decipher the probable origin of bacterial wilt of cardamom by assigning allele numbers after sequence analysis. We have adopted the experimental methodology suggested by Castillo and Greenberg (2007) for PCR amplification and sequencing of loci. Sequences were assembled using Vector NTi (Invitrogen) software package and sequences were subjected to two independent analyses. In the first analysis, forward and reverse sequences of the house-keeping and virulence genes from *R. solanacearum* from cardamom, ginger, paprika and potato were assembled and each of the sequences was subjected to blast analysis in http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl. The sequences from all eight loci were concatenated (420 bp+774 bp+873 bp+774 bp+717 bp+318 bp+686 bp+810 bp=5,372 bp) and subjected to multiple alignment. A phylogenetic tree was then constructed using neighbour-joining. The cardamom strain clustered with ginger strain whereas the other two strains formed two independent clades (Fig. 3). We further used the MLST scheme to assign different allele numbers to the sequences at each locus, getting unique allelic profile, called STs. Allele numbers were obtained by sequence comparison with allelic sequences presently contained in the database (Almeida et al. 2010). The isolate from cardamom yielded an identical allelic profile to that of the strain obtained from ginger indicating their close similarity (Table 2). Of the eight alleles, six of them were found to be novel for cardamom and ginger strains of *R. solanacearum*. After allelic profiles or sequence types (ST) were assigned to strains, these ST were compared with the ST of strains presently available in http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl using eBurst (http://eburst.mlst.net) (Feil et al. 2004, Spratt et al. 2004). The following settings for the

Table 2 Allelic profile of *Ralstonia solanacearum* causing bacterial wilt of cardamom

Loci	GenBank accession number	Allele length (bp)	Host origin of *Ralstonia solanacearum*			
			Ginger *Strain ID-912	Cardamom Strain ID-911	Paprika Strain ID-916	Potato Strain ID-915
*pps*A (Phosphoenol pyruvate synthase)	JF523197	717	10	10	25	2
*fli*C (Flagellin protein)	JF523192	318	19	19	22	2
*hrp*B (Regulatory transcription Regulator)	JF523196	810	27	27	28	2
*gap*A (Glyceraldehyde 3-phosphate dehydrogenase oxidoreductase)	JF523193	774	27	27	25	2
*gdh*A (Glutamate dehydrogenase Oxidoreductase)	JF523194	774	25	25	27	2
adk (Adenylate kinase)	JF523190	420	1	1	11	2
*gyr*B (DNA gyrase B)	JF523195	873	26	26	10	2
egl (Endoglucanase precursor)	JF523191	686	25	25	25	2
Concatenated sequence length=5,372 bp						

*For loci, 100% identity, no mismatches, no gaps, and an alignment over the full length of the locus were obtained. Alleles were obtained or assigned after consulting http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl as on 19th September 2011 where strain IDs are also available

creation of the eBURST-based snapshot were used: number of loci per isolate, 8; minimum number of identical loci for group definition, 0; minimal single-locus variant count for subgroup definition, 3; and number of re-samplings for bootstrap analysis, 1,000. In a eBurst diagram showing the population of 46 strains of STs of *R. solanacearum*, strain infecting cardamom (Strain ID 911) was clustered with ginger strain (Strain ID 912) indicating their strong association (data not shown).

The identical allelic profile observed for the strains collected in two different time points and geographical & host origin clearly confirms the prevalence of a single virulent lineage of *R. solanacearum* on cardamom and ginger in India. Widespread prevalence of such a virulent lineage of *R. solanacearum* has been attributed to its rhizome transmission across the diverse geographical locations in India (Kumar et al. 2004). Bacterial wilt of Zingiberaceae plants has been reported predominantly in edible ginger-*Zingiber officinale* in India (Kumar and Sarma 2004), Australia (Hayward et al. 1967), China (Ren et al. 1981), Malaysia (Lum 1973), Mauritius (Orian 1953), Philippines (Zehr 1970), Thailand (Titatarn 1986) and in Hawaii (Quinon et al. 1964). Other members of the Zingiberaceae family found to be host for the bacterium includes *Curcuma longa* in Sri Lanka (Velupillai 1986), *Hedychium* in Hawaii (Aragaki and Quinon 1965), *Kaempferia galanga* in China (He 1986), *Zingiber mioga* in Japan (Tsuchiya et al. 2005). Besides these natural incidences, *R. solanacearum* of ginger origin was found to wilt members of the Zingiberaceae such as *Alpinia galanga, Curcuma aromatica, C.longa*, *C. zedoria, Elettaria cardamomum, Globba* sp*, Kaempferia galanga,* and *Zingiber zerumbet* (Kumar and Hayward 2005; Kumar et al. 2006). Records on *R. solanacearum*-induced bacterial wilt in crop plants, particularly among the Zingiberaceae family, reveal that this is a new report of bacterial wilt disease in small cardamom. Apart from striking phenotypic and genotypic (allelic) similarities, geographical origin of the disease, the pathogenic behaviour, and cross transmissibility of the cardamom strain of *R. solanacearum* in cardamom and ginger strongly suggest that the new incidence of bacterial wilt of cardamom in India could have origin in bacterial wilt of ginger. Most likely the close proximity of ginger cultivations and cardamom plantations in the Wyanad district of Kerala, a location where bacterial wilt is endemic, could have enabled the ginger pathogen to infect cardamomin India.

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