Broad Diversity of *Ralstonia solanacearum* Strains in Cameroon

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ABSTRACT

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In 2005, an extensive survey of bacterial wilt in Cameroon collected 110 strains of *Ralstonia solanacearum* from wilting tomato, potato, pepper, huckleberry (*Solanum scabrum*), sesame, and amaranth. The genetic diversity and phylogeny of selected strains from Cameroon were assessed by multiplex-polymerase chain reaction (PCR), race 3/biovar 2-specific PCR, and sequence analyses of the *mutS* and *egl* genes. These data were compared with those from 33 reference strains covering the known diversity within the *R. solanacearum* species complex. Strains isolated in Cameroon clustered into three of the four known phylotypes: I (Asian), II (American), and III (African). Lowland tomato strains belonged to phylotype I and were quite homogeneous. The strains belonging to phylotype II were genetically diverse, and partitioned into subclusters IIA and IIB (sequevar 1, race 3/biovar 2). Cameroon strains in the African phylotype III were distinct from reference strains from Zimbabwe or the Indian Ocean, highlighting the genetic diversity present within this phylotype. Strains from potatoes growing in the highlands of West Cameroon fell into both phylotypes II (race 3/biovar 2) and III. These phylotype II and III highland strains attacked both potato and tomato and could therefore pose an economic threat to potato and tomato crops throughout Central Africa. This is the first comprehensive report on the genetic diversity of *R. solanacearum* strains in Cameroon.

Bacterial wilt, the vascular disease caused by the soilborne bacterium *Ralstonia solanacearum*, is regarded as a major limiting factor for many crops worldwide, including subsistence food crops, small farming cash crops, and industrial crops including vegetables (*Solanum tuberosum*, *S. lycopersicum*, *Capsicum* spp.), banana (*Musa* spp.), peanuts (*Arachis hypogaea*), and tobacco (*Nicotiana tabacum*) (15). Global economic losses due to bacterial wilt of potatoes have been estimated at US\$950 million per year (5). This disease,

also known as potato brown rot, is caused by the biovar 2 Andean subgroup of *R. solanacearum*, historically known as race 3 biovar 2 (R3bv2). R3bv2 is a quarantine pest in Europe and North America, and is listed in the United States as a bioterrorism Select Agent (20). In Africa, losses due to this pathogen have a major socio-economic impact on key resource crops such as tomato, pepper, eggplant, and potato (43), which are a source of cash for thousands of low-income farmers, especially in Cameroon (11). Bacterial wilt severely affects huckleberry (*Solanum scabrum*), also called African nightshade, an indigenous leafy vegetable widely grown in the humid forest zone of West and Central Africa as a subsistence food crop and cultivated for its edible leaves in more than 20 African countries (11).

Many different strategies have been evaluated for the control of bacterial wilt. These include soil disinfection (15), soil amendment (21,22,34), biological control (30,36), chemical elicitors of systemic acquired resistance (26), and the use of genetic resources such as resistant rootstock for grafting of susceptible varieties (12). To date, breeding for resistance to bacterial wilt is still considered the most

promising strategy for long-lasting control (29). Unfortunately, wilt resistance often breaks down due to the genomic plasticity and the large genetic and phenotypic diversity within the *R. solanacearum* species complex (6,10). For example, tomato lines that were resistant to bacterial wilt in one geographic area were susceptible in another because of local pathogen strain variation (40). In addition, the effectiveness of genetic resistance is highly dependent on the subtle interactions among host, pathogen, and environmental conditions (14,29,40,41). Thus, knowledge of local pathogen diversity is a key prerequisite for successful disease resistance breeding.

Historically, *R. solanacearum* was subclassified into races and biovars, but these systems were neither predictive nor phylogenetically meaningful. Recently, Fegan and Prior (6) proposed a new hierarchical classification scheme, based on sequence analysis of the internal transcribed spacer (ITS) region, the endoglucanase (*egl*) gene, and the *hrpB* gene, that subdivides *R. solanacearum* into phylotypes, defined as “a monophyletic cluster of strains revealed by phylogenetic analysis of sequence data” (6,7). Four phylotypes were distinguished. Phylotype I corresponds to the “Asiaticum” division 1 of Cook et al. (3) and contains strains belonging to biovars 3, 4, and 5. Phylotype II corresponds to the “Americanum” division 2 of Cook et al. (3) and contains strains belonging to biovar 1/race 1, biovar 1/race 2 (Moko disease-causing strains), R3bv2, and biovar 2T strains. Phylotype III contains strains from Africa and the Indian Ocean, which belong to biovars 1 and 2T. Phylotype IV contains strains from Indonesia, some strains from Japan, and a single strain from Australia, belonging variously to biovars 1, 2, and 2T. Phylotype IV also contains the closely related species *Ralstonia syzygii* and the banana blood disease bacterium (BDB). Each phylotype can be further subdivided into sequevars based on differences in sequence of a 750-bp region of the endoglucanase (*egl*) gene. The phylogenetic scheme proposed by Fegan and Prior (6) is broadly consistent with the former phenotypic and molecular typing

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schemes, and adds valuable information about the geographical origin and in some cases the pathogenicity of strains. A recent study (13) hybridized genomic DNA of 18 strains, representative of the known diversity within the *R. solanacearum* species complex, to a microarray of genes from the sequenced genome of strain GMI1000. The resulting hierarchical clustering of strains perfectly matched the previous classification in four phylotypes.

To date, although bacterial wilt disease is known to be widespread in Africa, the genetic diversity of *R. solanacearum* strains prevalent on the continent, and in Cameroon in particular, is scarcely documented. Strains were reported and described from potato crops in Kenya, Burundi, Nigeria, Egypt (35), South Africa (37), and Ethiopia (18), from tobacco crops in Zimbabwe (31), from eucalyptus in South Africa (4,9), from groundnut in Uganda (1), and from tomato in Benin (42). However, “there is little or no published information on distribution or importance” (5).

Understanding local pathogen diversity is the foundation of a successful breeding and integrated management program (33). In Cameroon, a breeding program was launched to improve bacterial wilt resistance in pepper. The purpose of this present study was to assess the overall genetic diversity of *R. solanacearum* that may challenge new pepper germplasm. The resulting distribution and diversity map of the pathogen populations will be used to optimize this pepper breeding program, but also to focus African bacterial wilt control strategies in Solanaceous crops, including potatoes.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study ($n = 143$) are listed in Table 1 and Supplemental Table 1. They include *R. solanacearum* strains from Cameroon ($n = 110$) and international reference strains ($n = 33$) provided by authors and obtained from collections (Collection Françaises de Bactéries Phytopathogènes [CFBP], Angers, France).

Strains were collected throughout Cameroon in March 2005 during an extensive survey covering a linear distance of about 2,500 km. Fifty-two sites were identified by local or regional extension agents and surveyed for sampling. Various host species of economic importance were sampled, including tomato (*Solanum lycopersicum*), sweet pepper (*Capsicum annuum*), hot pepper (*Capsicum chinense*), potato (*Solanum tuberosum*), huckleberry (*S. scabrum*), amaranth (*Amaranthus* spp.), and sesame (*Sesamum* spp.). Two priorities guided the survey: (i) representing the five different agro-ecological zones (AEZ) present in Cameroon (Table 2), and (ii) sampling the major vegetable cropping areas within each AEZ where bacterial wilt

was known to be the major biotic constraint to crop production. Most fields had a single collection site, but some large fields were sampled at two sites. At each collection site, two or three plants showing typical bacterial wilt symptoms were cut at midstem, and stem pieces (6 to 10 cm long) were kept at about 20°C in a cool box prior to isolation of *R. solanacearum*. Sampling and isolations were done on the same day. Samples were surface-disinfected with 70% ethanol, peeled, subsampled, and macerated in sterile distilled water. Macerates were streaked on Kel-

man's triphenyltetrazolium chloride (TZC) agar medium (17) supplemented with 1% yeast extract. Plates were incubated at room temperature ($28 \pm 5^\circ\text{C}$). Bacterial colonies developing the typical irregular mucoid *R. solanacearum* morphotype were then subcultured on fresh TZC medium for further purification. Isolated strains of *R. solanacearum* were given an isolate number and stored in sterile water at room temperature.

A set of 44 representative *R. solanacearum* strains from Cameroon, chosen on the basis of their phylotype assignment, host

Table 1. *Ralstonia solanacearum* strains used in this study

Strain ^a	Host	Origin (AEZ) ^b	Phylotype/sequovar ^c	Pathogenicity tested ^d	Reference ^e
Strains from Cameroon					
CMR1	Tomato	Obala (V)	I/13		TS
CMR7	Tomato	Obala (V)	I/13		TS
CMR13	Tomato	Obala (V)	I/13		TS
CMR134	Huckleberry	Bafia (V)	I/13	YES	TS
CMR137	Tomato	Bafia (V)	I/13		TS
CMR96	Huckleberry	Akonolinga (V)	I/13		TS
CMR117	Tomato	Yaoundé (V)	I/13		TS
CMR109	Tomato	Yaoundé (V)	I/13		TS
CMR111	Amaranthus	Yaoundé (V)	I/13		TS
CMR128	Tomato	Yaoundé (V)	I/13		TS
CMR17	Tomato	Obala (V)	I/14		TS
CMR123	Tomato	Yaoundé (V)	I/14		TS
CMR87	Tomato	Akonolinga (V)	IIA/35		TS
CMR89	Tomato	Akonolinga (V)	IIA/35		TS
CMR91	Huckleberry	Akonolinga (V)	IIA/35		TS
CMR102	Tomato	Akonolinga (V)	IIA/35		TS
CMR120	Tomato	Yaoundé (V)	IIA/35	YES	TS
CMR131	Tomato	Yaoundé (V)	IIA/35		TS
CMR116	Tomato	Yaoundé (V)	IIA/35		TS
CMR118	Tomato	Yaoundé (V)	IIA/35		TS
CMR47	Tomato	Foumbot (III)	IIA/35		TS
CMR69	Tomato	Bafang (III)	IIA/35		TS
CMR39	Tomato	Dschang (III)	IIA/41	YES	TS
CMR121	Tomato	Yaoundé (V)	IIA/52		TS
CMR18	Tomato	Baham (III)	IIB/1		TS
CMR24	Potato	Bamougoum (III)	IIB/1	YES	TS
CMR34	Tomato	Baham (III)	IIB/1	YES	TS
CMR35	Tomato	Baham (III)	IIB/1	YES	TS
CMR44	Potato	Dschang (III)	IIB/1		TS
CMR15	Tomato	Obala (V)	III/29	YES	TS
CMR30	Tomato	Baham (III)	III/29		TS
CMR32	Huckleberry	Dschang (III)	III/29		TS
CMR36	Tomato	Baham (III)	III/29		TS
CMR43	Potato	Dschang (III)	III/29	YES	TS
CMR50	Tomato	Mbouda (III)	III/29		TS
CMR51	Tomato	Mbouda (III)	III/29		TS
CMR52	Potato	Dschang (III)	III/29		TS
CMR56	Huckleberry	Foumbot (III)	III/29		TS
CMR62	Tomato	Foumbot (III)	III/29		TS
CMR63	Tomato	Foumbot (III)	III/29		TS
CMR75	Tomato	Melong (IV)	III/29		TS
CMR76	Tomato	Melong (IV)	III/29		TS
CMR78	Pepper	Melong (IV)	III/29	YES	TS
CMR66	Huckleberry	Bafang (III)	III/49		TS

(continued on next page)

^a Names of strains in Reunion collection (strains from Cameroon) and in international collections (references). CFBP: Collection Française de Bactéries Phytopathogènes [CFBP], Angers, France; RUN: collection at CIRAD-INRA Reunion.

^b AEZ = agro-ecological zone (Cameroon).

^c Phylotypes were determined by phylotype multiplex-polymerase chain reaction (Pmx-PCR). II/1 strains amplified the race3/biovar2 (R3bv2)-specific band with primer pair 630/631. The phylotype was confirmed by phylogenetic trees based on *mutS*, and sequovars were determined by phylogenetic trees based on *egl* sequence analyses as described in Fegan and Prior (6,7) and Prior and Fegan (28).

^d Pathogenicity tested on potato, tomato, and pepper (see results in Table 4).

^e TS = this study; I = P. Prior, INRA-CIRAD, Reunion Island, unpublished data.

of isolation, and geographical origin, were deposited at the Collection Française de Bactéries Phytopathogènes [CFBP] in Angers, France (Supplemental Table 2).

Pathogenicity tests. A set of seven *R. solanacearum* Cameroon strains and seven reference strains that spanned the genetic diversity reported in this study was tested for pathogenicity on susceptible cultivars of potato (cv. Désirée), tomato (cv. L390), and Pepper (cv. Yolo Wonder) in a growth chamber at 28 ± 1°C day/24 ± 1°C night, relative humidity of 90 ± 5% and a 14 h/10 h photoperiod. Bacterial suspensions were prepared in Tris buffer (0.01 M Tris/HCl, pH 7.2) and spectrophotometrically ad-

justed to OD_{600nm} = 0.1 (approximately 10⁸ CFU ml⁻¹). The root system of each plant was wounded with a scalpel; then 5 ml of inoculum per plant was poured on the wounded root system of 10 plants belonging to each of the three susceptible plant species at the three to four fully expanded leaf stage. Ten plants of each species, drenched with 0.01 M Tris buffer (pH 7.2), were used as controls. Symptom development in inoculated plants was monitored every 4 to 5 days for 4 weeks after inoculation. If at least one plant showed typical wilt symptoms, the interaction was classified as compatible (pathogenic).

Table 1. (continued from preceding page)

Strain ^a	Host	Origin (AEZ) ^b	Phylotype/ sequevar ^c	Pathogenicity tested ^d	Reference ^e
Reference strains					
R292	Mulberry	China	I/12		(27)
JT523	Potato	Reunion Is.	I/13		(27)
CFBP765	Tobacco	Japan	I/15		(27)
ACH92	Ginger	Australia	I/16		(27)
GMI1000	Tomato	French Guyana	I/18		1
CFBP2968	Eggplant	Guadeloupe	I/18		1
NCPBP3190	Tomato	Malaysia	I/18		1
CFBP2972	Potato	Martinique	IIA/35		1
CFBP2957	Tomato	Martinique	IIA/36		1
CFBP2958	Tomato	Guadeloupe	IIA/39		1
ICMP7963	Potato	Kenya	IIA/7		(27)
K60	Tomato	USA	IIA/7		(27)
IPO1609	Potato	Netherlands	IIB/1	YES	(27)
JT516	Potato	Reunion Is.	IIB/1	YES	(27)
UW551	Geranium	Kenya	IIB/1	YES	(38)
CIP10	Potato	Peru	IIB/25	YES	(27)
NCPBP3987	Potato	Brazil	IIB/28	YES	(27)
MOLK2	Banana	Philippines	IIB/3		(27)
UW9	Heliconia	Costa Rica	IIB/3		(27)
CFBP1409	Banana	Honduras	IIB/3		(27)
CFBP1183	Heliconia	Costa Rica	IIB/3		(27)
UW70	Banana	Colombia	IIB/4		(27)
UW129	Banana	Peru	IIB/4		(27)
UW162	Banana	Peru	IIB/4		(28)
CFBP6784	Anthurium	Martinique	IIB/4		(28)
A3909	Heliconia	Hawaii	IIA/6		(27)
UW21	Banana	Honduras	IIA/6		(28)
CFBP734	Potato	Madagascar	III/19		(28)
JT525	Geranium	Reunion Is.	III/19	YES	(28)
NCPBP332	Potato	Zimbabwe	III/22		1
CFBP3059	Eggplant	Burkina Faso	III/23	YES	1
CIP358	Potato	Cameroon (III)	III/29		1
MAFF301558	Potato	Japan	IV/8		1
PSI7	Tomato	Indonesia	IV/10		(27)

Table 2. Characteristics of agro-ecological zones (AEZ) in Cameroon

AEZ (districts)	Rainfall (mm)	Elevation (m.a.s.l.) ^a	Mean annual temp. (range)
I Sudano - sahelian zone (Garoua)	500-900	250-500	28°C (7.7)
II High Guinea savannah (Ngaoundéré)	1,500-1,800	500-1,500	23°C (6.4)
III Western highlands (Bamougoum, Foubot, Baham, Dschang, Mbouda)	1,800-2,400	1,500-2,500	21°C (2.2)
IV Humid forest (monomodal rainfall) (Melong, Buea)	2,000-11,000	0-2,500	26°C (2.8)
V Humid forest (bimodal rainfall) (Yaoundé, Okola, Obala, Bafia, Akonolinga)	1,500-2,000	400-1,000	25°C (2.4)

^a Meters above sea level.

DNA typing. Phylotype multiplex-polymerase chain reaction (Pmx-PCR) of the 16S-23S ITS was used to determine the phylotype of each strain based on the methods and hierarchical classification scheme described previously (6). Briefly, Pmx-PCR includes the primers 759/760 (23) as an internal marker to amplify the 280-bp “universal” *R. solanacearum*-specific reference band, plus a set of four phylotype-specific forward primers (Nmult:21:1F: 5'-CGTTGATGAGGCGCG CAATTT-3'; Nmult:21:2F: 5'-AAGTTA TGGACGGTGAAGTC-3'; Nmult:22:1nF: 5'-ATTGCCAAGACGAGAGAAGTA-3'; Nmult:23:AF: 5'-ATTACGAGAGCAATC GAAAGATT-3') with a unique and conserved reverse primer (Nmult:22:RR: 5'-TCGCTTGACCCTATAACGAGTA-3'). This Pmx-PCR produces the following phylotype-specific PCR products: a 144-bp amplicon from phylotype I strains; a 372-bp amplicon from phylotype II strains; a 91-bp amplicon from phylotype III strains; and a 213-bp amplicon from phylotype IV strains. Strains that were classified as phylotype II were further tested using the PCR primer pair 630-F (5'-ATACAG AATTCGACCGGCACG-3') and 631-R (5'-AATCACATGCAATTCGCCTACG-3'), which specifically amplifies a 357-bp fragment from strains in phylotype II, sequevar 1, historically known as R3bv2 (8).

PCR amplification and DNA sequencing of the endoglucanase (*egl*) and DNA repair (*mutS*) genes. Strain genetic diversity and phylogeny were further characterized by comparative analysis of the partial nucleotide sequences of the endoglucanase (*egl*) and the DNA repair protein (*mutS*) genes. PCR amplifications of a 750-bp region of the *egl* gene were performed using the primer pair Endo-F (5'-ATGCATGCCGCTGGTCGCCGC-3') and Endo-R (5'-GCGTTGCCCGGCACGAAC ACC-3') (6). The reaction mixture (50 µl, total volume) contained 1× PCR buffer (supplied by the manufacturer), 1.5 mM MgCl₂, 200 µM of each dNTP, 4 pmol of each primer, a 2 µl volume of a turbid bacterial suspension as template (about 5 ng/µl), and 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). Reaction mixtures were heated to 96°C for 9 min and then cycled through 30 cycles of 1 min at 95°C, 40 s at 55°C, 2 min at 72°C, with a 10 min final extension at 72°C, and a final hold at 12°C. Amplification of the 758-bp fragment of the *mutS* gene was performed in a total volume of 25 µl containing 1× PCR buffer (Promega, Charbonnières-les-Bains, France), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.8 µM DMSO, 1.25 U of *Taq* Polymerase (Promega), 9 pmol of the primers mutS-RsF.1570 (5'-ACAGCGCCTTGAGCCGGT ACA-3') and mutS-RsR.1926 (5'-GCT GATCACCGGCCCGAACAT-3'), and 2 µl of a turbid bacterial suspension as template (about 5 ng/µl). Reaction mixtures were

first heated to 96°C for 5 min, and then cycled through 35 cycles of 1 min at 94°C, 1 min at 66°C, and 1.5 min at 72°C, with a 5-min final extension at 72°C (27). All

PCR amplifications were performed using an MJ PTC100 thermocycler (MJ Research, Bio-Rad Laboratories, Inc., Hercules, CA). Samples (5 µl) of PCR reaction

mixtures were resolved on a 2% Tris-acetate-EDTA agarose electrophoresis gel, and PCR products were revealed by staining DNA in 0.5 µg·ml⁻¹ ethidium bromide. PCR products were sent to MWG-biotech (Ebersberg, Germany) for purification and sequencing of both strands. For both *egl* and *mutS* genes, PCR primers used to amplify partial sequence of these genes were also used as sequencing primers.

Sequence analysis and construction of phylogenetic trees. The partial sequences of the chromosomally located housekeeping gene *mutS* and the megaplasmid-located virulence gene *egl* were analyzed using the ARB Software Environment (19) (<http://www.arb-home.de/>). Sequences were manually aligned using the ARB sequence editor. Evolutionary relationships between sequences were analyzed using the three major phylogenetic approaches offered by the ARB Software: (i) using the algorithm of Jukes and Cantor (16), phylogenetic trees were constructed from the genetic distance data by using the neighbor-joining (NJ) method (32) with 5,000 bootstrap resamplings of the data to test the tree topologies; (ii) maximum likelihood (ML) methods using the FastDNAmI program; and (iii) the maximum parsimony (MP) methods using the Phylip DNAPARS package implemented by the ARB software. From the pool of *mutS* and *egl* sequences included in this study (77 and 76 sequences, respectively), representative reference strains were identified, and sequences from newly described strains were deposited into the GenBank database.

RESULTS

***R. solanacearum* strain collection.** A total of 110 *R. solanacearum* strains were recovered from wilted tomato, sweet and hot pepper, potato, huckleberry, amaranth, and sesame plants (Table 1). Bacterial wilt is widely distributed in Cameroon, but disease incidence and severity were highest in AEZ V (Yaoundé, lowlands) and AEZ III (Bafoussam, highlands), which are also the main vegetable cropping areas of Cameroon. The phylotyping results of the 110 *R. solanacearum* strains by AEZ are reported in Table 3. From these 110 strains, a set of 44 representative strains collected in Cameroon was selected for phylogenetic analysis (Table 1), including strains from all phylotypes and most of the hosts. No *R. solanacearum* strains were obtained from AEZ I (Fig. 1).

PCR-based identification and characterization of *R. solanacearum* strains. Multiplex PCR analyses demonstrated that three of the four *R. solanacearum* phylotypes were present in Cameroon: the Asian phylotype I, the American phylotype II, and the African phylotype III (Table 4). Strains of phylotype I constituted the largest group (45.5% of the 110 strains). Strains of phylotype II represented 35% of the collection. Among these phylotype II

Table 3. Phylotype distribution of *Ralstonia solanacearum* strains collected in Cameroon within agro-ecological zones (AEZ) based upon DNA typing using phylotype multiplex-polymerase chain reaction (PCR) and the race 3/biovar 2 (R3bv2)-specific 630/631 PCR tests

AEZ	No. of strains				Total no. of strains
	Phylotype I	Phylotype II subcluster/sequevar		Phylotype III	
		A/non-R3bv2	B/1 (R3bv2)		
II	0	1	0	0	1
III	0	11	5	14	30
IV	0	1	0	5	6
V	50	22	0	1	73
Total no. of strains (%)	50 (45.5)	35 (31.8)	5 (4.5)	20 (18.2)	110
Hosts of isolation	Tomato	Tomato	Tomato	Tomato	
	Potato	Pepper	Potato	Potato	
	Pepper	Huckleberry		Pepper	
	Huckleberry			Huckleberry	
	Amaranth

Fig. 1. Map of Cameroon showing the five agro-ecological zones and the locations where *Ralstonia solanacearum* strains of phylotype I (stars), IIA (open circles), IIB (closed circles), and III (open squares) were sampled.

strains, 5% were identified as sequevar 1 (R3bv2) because the characteristic R3bv2-specific 304-bp band was amplified from their DNA using the 630/631 primer pair. Strains of African phylotype III represented only 18% of the 110 collected isolates.

Pathogenicity. All Cameroon strains tested ($n = 7$) were pathogenic on potato (Table 4). Plants developed symptoms approximately 5 days after inoculation, showing a typical leaf margin rolling-up and epinasty, followed by a generalized and characteristic wilt. All strains except the reference R3bv2 strain IPO1609 (13.39) were pathogenic to tomato. Cameroon strains CMR15 and CMR43, as well as reference strains IPO1609, CIP10, and NCPPB3987, were classed as not pathogenic to pepper because no symptoms developed 4 weeks after inoculation.

Phylogenetic analysis. Partial *egl* and *mutS* gene sequences were generated from a set of representative strains from Cameroon ($n = 44$) and compared with those from *R. solanacearum* reference strains covering the known genetic diversity within the four phylotypes (6,27). Trees constructed with maximum likelihood, parsimony, and distance NJ phylogenetic approaches were totally congruent; therefore, we present only NJ phylogenetic trees with bootstrap values (Figs. 2 and 3). Trees constructed with *egl* and *mutS* sets of sequences were in general agreement with one difference in topology: phylotype III was most closely related to phylotype II in the *egl*-derived tree, while it was more closely related to phylotype I in the *mutS* tree. Apart from this different branching order, trees constructed using the two different sequences showed similar branching patterns clearly partitioning the four phylotypes as previously described (6). The reference strains from phylotypes I, II, III, and IV clustered separately with high bootstrap values.

The 44 representative strains collected in Cameroon were classified into phylotypes

according to Pmx-PCR results (Table 1). Phylotype I strains were isolated from various hosts including tomato, *Solanum nigrum* (CMR96 and CMR134), and *Amaranthus* spp. (CMR111) produced in the hot and humid lowland AEZ V. These strains were closely related according to both *egl* and *mutS* sequence analyses.

Phylotype II strain DNA sequences resolved into two main branches: subclusters A and B (7). Within subcluster A, all strains isolated from tomato and huckleberry had *egl* sequences differing by less than 1% from the *egl* sequence of sequevar 35 reference strain CFBP2972 (P. Prior, INRA-CIRAD, Réunion, unpublished data). They were thus assigned phylotype IIA sequevar 35 (IIA/35). Within subcluster B, all strains were indistinguishable from reference sequevar 1 strains IPO1609 (The Netherlands) and JT516 (Reunion Island) (27). These phylotype IIB/1 strains were all isolated in the cool highlands of AEZ III from potato plants with brown rot symptoms (CMR24, CMR44) and from wilting tomato plants (CMR18, CMR34, CMR35).

Phylotype III strains from Cameroon fell, for most of these, in a subgroup near the reference strain CIP358, previously typed as phylotype III/29 (P. Prior, INRA-CIRAD, Réunion, unpublished data). The strain CMR66 was more closely related to the phylotype III/23 reference strain CFBP3059 from Burkina Faso (6). No strains collected in Cameroon in this study belonged to phylotype IV, the Indonesian cluster of *R. solanacearum*.

Geographic distribution of phylotypes in Cameroon. The distribution of phylotypes within the agro-ecological zones visited in this study reflected some epidemiological traits of *R. solanacearum* strains (particularly regarding virulence/temperature relationships) (Table 3). *R. solanacearum* was mainly isolated in the humid lowland AEZ V and in the highland AEZ III. Phylotype III strains were

found in AEZ III, IV, and V, with a majority collected in AEZ III, which is a major potato and tomato cropping area. Phylotype II strains were found in AEZ II, III, and IV; however, phylotype IIB strains were more prevalent in the highlands of West Cameroon (AEZ III), whereas phylotype IIA strains, isolated from various hosts, were distributed among all AEZs visited. All phylotype I strains were collected in the lowland AEZ V (see Figure 1 and Table 2 for AEZ characteristics).

DISCUSSION

Bacterial wilt, caused by *R. solanacearum*, was observed in the major vegetable cropping areas of highland and lowland regions surveyed in Cameroon. In this country, farmers from all the regions visited were familiar with bacterial wilt, called “Djom-djom” disease in the Obala area (AEZ V), literally translated as “sleep-sleep” referring to the sudden wilt of tomatoes; “Sudden-death” in Baham (AEZ III); “Water-disease” in Foubot (AEZ III) in the highlands of West Cameroon; and “Direct-attack” by tomato growers in Buea (AEZ IV) on the slopes of Mount Cameroon. One hundred and ten strains of *R. solanacearum* were collected from the major cash and/or subsistence crops, and 44 representative strains were positioned in the global phylogenetic scheme of the *R. solanacearum* species complex (6), along with 33 reference strains. These analyses revealed a high genetic diversity among the pathogen strains collected in this country. Phylotype assignment by Pmx-PCR was perfectly congruent with phylogenetic positioning based on sequences of *egl* and *mutS* genes.

Cameroon strains of *R. solanacearum* belonged to three of the four previously described phylotypes (6): I, II, III. The presence in Africa of these three groups was previously known (5), but our study is the first to describe the distribution of the three phylotypes in a single Central Afri-

Table 4. Pathogenicity of selected *Ralstonia solanacearum* strains on potato, tomato, and pepper

Strain ^a	Host	Origin ^b	Phylotype and subcluster ^c	Pathogenicity on		
				Potato	Tomato	Pepper
CMR134	Huckleberry	Bafia (V)	I	+	+	+
CMR39	Tomato	Dschang (III)	IIA	+	+	+
CMR120	Tomato	Yaoundé (V)	IIA	+	+	+
CMR34	Tomato	Baham (III)	IIB	+	+	+
JT516	Potato	Reunion Is.	IIB	+	+	+
UW551	Geranium	Kenya	IIB	+	+	+
IPO1609	Potato	Netherlands	IIB	+	-	-
NCPB3987	Potato	Brazil	IIB	+	+	-
CIP10	Potato	Peru	IIB	+	+	-
CMR43	Potato	Dschang (III)	III	+	+	-
CMR15	Tomato	Obala (V)	III	+	+	-
CMR78	Pepper	Melong (IV)	III	+	+	+
JT525	Geranium	Reunion Is.	III	+	+	+
CFBP3059	Eggplant	Burkina Faso	III	+	+	+
Control (water)				-	-	-

^a Strains from Cameroon (CMR) and references.

^b District (agro-ecological zone) in Cameroon or country of origin.

^c Phylotypes and subclusters were determined by *egl* and *mutS* sequence analyses.

can country. Phylotype I strains of *R. solanacearum* represented almost half of the collected strains and were over-represented in AEZ V of Yaounde, a warm and humid lowland region, especially in the rainy season of March (Table 4). This result confirms previous reports on phylotype I (ex-biovar 3) strains originating from Southern Africa (24,25). As expected from this group, they were found in several host species including tomato, potato, pepper, huckleberry, and amaranth. Phylotype I strains may thus be considered the major bacterial wilt constraint for vegetable crops in the lowland bimodal humid forest region of Cameroon (AEZ V; Yaoundé, Akonolinga, Obala, Bafia).

In contrast, phylotype III and phylotype IIB (R3bv2) strains were preferentially isolated from AEZ III, a highland cropping area with a temperate climate (Baham,

Bafoussam, Bamougoum, Dschang, Fombot). Strains from phylotype IIA were found in both AEZ III and AEZ V. A further survey including a systematic collection sampling method may help to refine and validate these geographic distribution patterns.

Among the phylotype II strains of *R. solanacearum*, we noted differing host range and geographical distributions for IIA and IIB strains. Phylotype IIA strains were isolated from, and prevalent on, tomato and huckleberry crops in all regions sampled, with a majority of the strains originating from the lowland areas. Phylotype IIB/1 (R3bv2) strains were only isolated from potato and tomato in the highland AEZ III, the major potato cropping region. These strains, which are considered to be near-clonal, are hypothesized to have adapted (coevolved) with potato (10). This

study confirms that strains in phylotype IIB/1 have a host range that is not limited to potato under natural African highland and tropical conditions. This finding is of major concern for tomato growers in Cameroon, as well as in other African countries where phylotype II/1 strains (R3bv2) have been reported, including Nigeria (35), Uganda, Rwanda, and Tanzania (5), Ethiopia (18), and South Africa (5,9). Indeed, some phylotype IIB/1 strains are highly virulent on the most commonly used wilt-resistant tomato lines (2).

We show here that phylotype III strains of Cameroon have the same host range (potato and tomato) and the same cool highland environment (AEZ III) as phylotype IIB/1 strains, and caused the same brown rot-like syndrome on potato. Although indistinguishable on the basis of symptom development, disease severity in

Fig. 2. Phylogenetic neighbor-joining tree based on the partial endoglucanase (*egl*) gene sequences of strains from Cameroon and *Ralstonia solanacearum* species complex reference strains. The number at each node is the bootstrap value (5,000 resamplings), and significant bootstrap values less than 100% are indicated at each node. Scale bar represents 1 nucleotide substitution per 100 nucleotides.

potato, and prevalence in cool potato growing areas, *R. solanacearum* strains that cause wilt in potato in the highland areas of Cameroon should be referred to as (i) the African brown rot group, composed of phylotype III strains, and (ii) the Andean brown rot group, composed of phylotype IIB/1 (R3bv2) strains that most likely were introduced through imported latently infected potato tubers. The presence of these two phylogenetically distinct but epidemiologically congruent pathogen groups in the West African highlands must be taken into account in the development of control strategies, including diagnostic tools and especially regional disease resistance breeding programs.

Phylogenetic analysis of *R. solanacearum* strains collected in Cameroon and comparisons with reference strains from neighboring countries revealed that the recently described phylotype III (6), formerly described as the "African biovar 1" strains (24,25), encompasses broad vari-

ability. Three subgroups with strong bootstrap support were observed, distinguishing (i) the southern Africa and Indian Ocean strains (III/19 and II/21), containing the reference strain NCPB332 (from Zimbabwe), and phylotype III/19 reference strains CFBP734 (Madagascar) and JT525 (Reunion Island), (ii) the Central Africa strains, mostly represented in Cameroon (III/29), and (iii) a subgroup still poorly investigated, containing the strain CMR66, and the phylotype III/23 reference strain CFBP3059 from Burkina Faso (6). We thus tentatively propose further subdivision of phylotype III into three clusters, awaiting further data from additional African strains for confirmation. Additional pan-African studies in southern, central, and northern Africa should reveal the genetic variability that remains to be described within phylotype III.

As a follow-up to this study, a typical lowland phylotype III strain, CMR15 (CFBP6941), is being completely se-

quenced to investigate the conserved and specific gene repertoires of these hitherto poorly described strains within the *R. solanacearum* species complex.

This study provides the first comprehensive information on genetically diverse strains of *R. solanacearum* in the Central African country of Cameroon. A recent survey in Ethiopia (18), although based on biovar classification, suggests that the diversity we found in Cameroon is not unique in Africa. The relationships between the wide genetic diversity and strain virulence phenotypes demands further investigation. Understanding the biological implications of such broad genetic diversity is critically important for the development of locally adapted strategies for bacterial wilt resistance breeding in Cameroon and other African countries that share similar agro-ecological environments.

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Fig. 3. Phylogenetic neighbor-joining tree based on partial sequences of the DNA repair *mutS* gene from strains from Cameroon and *Ralstonia solanacearum* species complex reference strains. The number at each node is the bootstrap value (5,000 resamplings), and significant bootstrap values less than 100% are indicated at each node. Scale bar represents 1 nucleotide substitution per 100 nucleotides.

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