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First phylotype analysis of *Ralstonia solanacearum* causing Eggplant bacterial wilt in the Republic of Guinea

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1 ABSTRACT

Eggplant is one of the important cash crops in Guinea and it is cultivated in all the agro-ecological zones of the country with the areas and production are constantly growing. However, this crop is affected by bacterial wilt disease caused by bacteria of the *Ralstonia solanacearum* species complex (RSSC). in these production areas. In August 2018, a total of 81 strains were collected from stems of withered eggplant plant in the nine prefectures of the three administrative regions of Guinea. These strains were extracted and put on FTA™ cards at the Foulayah laboratory in Guinea. for their molecular characterization the FTA cards were transported to the World vegetable center laboratory in Taiwan where all the 81 strains were confirmed as *R. solanacearum* . single 280 bp fragment resulted in all the isolates following polymerase chain reaction (PCR) amplification using the *R. solanacearum* specific universal primer pair 759/760. A phylotype specific multiplex PCR revealed that 55 of the 81 strains could be assigned to phylotypes. 85, 5% of this batch consisted of phylotype I; 3, 6% of phylotype II; 10, 9% of phylotype III. The objective of this study is to open the way to developing better eggplant BW management strategies for Guinea, including screening for eggplant lines resistant to phylotype I and phylotype III either together or separately for subsequent hybridization.

2 INTRODUCTION

Eggplant (*Solanum melongena*) is cultivated in all regions of Guinea. It is one of the most important crops because of the income it generates; it is most cultivated during the rainy season throughout the country hillsides and during the dry season in the lowlands (Fewst net, 2017). The *Solanum melongena* species is the most cultivated eggplant species; however, African eggplant species (*Solanum macrocarpon* and *Solanum aethiopicum*) are cultivated almost everywhere in Guinea as they are consumed in most dishes in various forms. In Guinea, statistical data on vegetable crops are non-existent because: (i) there is a great diversity of units of measurement (bags, piles.) depending on the zones, and the importance of self-consumption complicates the quantification of field data. (ii) Given the limited resources of the national agency for agricultural and food statistics (ANASA), these crops are

unfortunately not a priority compared to cereals and perennial crops (FAO, 2015). Eggplant is one of the most economical vegetable crops in the large urban areas of Guinea, (USAID, 2015). However, its production is affected by bacterial wilt in all production areas in Guinea. Bacterial wilt, caused by bacterium *R. solanacearum*, (*Pseudomonas solanacearum* EF Smith) is one of the most devastating and prevalent bacterial diseases of vegetables in tropical areas (Buyla *et al.,* 2017); She et al. 2017); Garcia 2019). Bacterium *R. solanacerum* cause bacterial wilt in a wide host range of more than 350 species in 54 botanical families, including monocots and dicots in the tropical, subtropical and warm regions of the world (Abebe *et al.,* 2020; Landry *et al.,* 2020). The pathogen penetrates the roots and colonizes the xylem vessels of its hosts. Typical visible symptoms above ground are rapid wilting of the foliage (Pawaskar *et al.,* 2014). Bacterial wilt is caused by the *R. solanacearum* species complex (RSSC). RSSC strains were initially subdivided into five "races" based on host range, and in five carbohydrate- based "biovars" (Fegan and Prior, 2005). Recently, using the sequence analysis of the internal transcribed spacer (ITS) region of the 16S - 23S rRNA gene (Fegan *et al.,* 1998), RSSC species have been divided into four phylogenetic groups. Phylotype I correspond to strains originating in Asia; American phylotype II strains; phylotype III includes strains from Africa and phylotype IV strains from Indonesia Prior and (Fegan, 2005; Wicker *et al.,* 2012). This phylotype IV harboured the closely related species *R. syzygii* and the bacteria of banana blood disease (BBD), even where the soil is moist (Meng, 2013). Recently the RSSC has been subdivided into three genomic species: *R.pseudosolanacearum* (phylotype I and III), *R. solanacearum* (phylotype II), and *R.syzygii* (phylotype IV, Blood Disease Bacterium, *R.syzygii*). In Africa, bacterial wilt is known to be widespread; however, the genetic diversity of the strains of *R. solanacearum* is not yet well documented (Chamedjeu *et al.,* 2018). In bordering countries of Guinea, race 1 biovars 3 has been reported in Mali on potato (Thera, 2010) and phylotype I and III were reported on African eggplant (Bihon *et al.,* 2020). In Sierra Leone and Senegal, except race 3, all phylotypes exist on Solanaceae (EPPO, 2004). In Côte d'Ivoire, phylotypes I, II and III have been reported on Solanaceae, (N'Guessan *et al.,* 2012). In Guinea, wilting is observed in all agroecological zones (ZAE) and reported by farmers. Few Guinean samples have been analysed by INRA France and phylotype III has been reported on potatoes. (Cellier *et al.,* 2010). Therefore, the present study aimed to use a phylotyping scheme to determine the phylotypes of strains of *R. solanacearum* 46 causing bacterial wilt of eggplant in Guinea.

3 MATERIALS AND METHODS

3.1 Survey and Sample Collection: The survey was carried out in the three regions of Guinea (Lower, Upper Guinea and Forest Guinea) in 2018, focusing on the main eggplant growing areas and localities where farmers and regional extension workers have already reported outbreaks of bacterial wilt. In total, nine prefectures in these agro-ecological zones (ZAE) (Table 1) were visited; in each prefecture, three or four fields were sampled; the geographic coordinates of each field were collected using a GPS (Altitude, Longitude, Latitude and Latitude).

Table 1: Characteristics of zones sampled in Guinea

N°	Prefectures (districts)	Climatic features[a]	Elevation (m.a.s.l)	Rainfall	Mean annual Temperature
1	Boke	Am	42	2513	27.5 (4.3)[b]
2	Coyah	Am	32	3537	27.9 (4.6)
3	Kindia	Aw	409	2202	25.7 (4.3)
4	Forecariah	Am	32	3244	29.1 (6.5)
5	Kankan	Aw	385	1545	26.0 (6.3)
6	Siguiri	Aw	505	1293	25.9 (6.4)
7	Dabola	Aw	431	1414	26.0 (6.7)
8	Macanta	Aw	546	2757	21.9 (3.4)
9	N'Zerekore	Aw	428	1027	24.1 (3.5)

[a]Am : Tropical Monsoon Climate; *Aw* = Tropical wet and dry or savana climate (https://en.climate-data.org/africa/guinea-46/).

3.2 Isolation and purification of Ralstonia species: stem samples of wilted or withering eggplant (*S. melongena*, S. *macrocarpon* and *S. aethiopicum*) suspected of being infected with BW were collected and carried to the laboratory for analysis. Stem pieces (6-10 cm long) were cut, washed thoroughly with water and then surface disinfected with 70% alcohol. Sub samples (5-10 mm) were peeled and the internal tissue macerated in sterile distilled water. Macerates were streak on Kelman's Triphenyltetrazolium chloride (TZC) agar medium, (Champoiseau, 2008). Plates were incubated at 28°C for three days. Pure bacteria colonies developing the typical irregular mucoid colonies were again streaked onto fresh modified medium by exclusion 60 of TZC for further purification. Isolated strains were labeled in Eppendorf tubes and stored in the distilled water in freeze for the further study.

Wilted plant observed 08/20/2018 in field in Kindia
(Photos GBonamou M.)

3.3 Whatman FTA®Elute card use for storage, transport and extraction of DNA: Using the method of Burlakoti *et al.,* (2019), Whatman FTA® Elute cards (Flinder Technology Associates) were used to capture, store, transport, and extract DNA from the strains of bacteria isolated from eggplant. Briefly, a small mass of each bacterial strain (about 10^4 CFU) from 2-3 day old TZC cultures was transferred to a separate micro-centrifuge tube containing 200 µl of 70% ethanol and 100 µl of each suspension was loaded to separate FTA card disks. The loaded cards were dried in a laminar flow hood (~2 hours) then stored in a desiccator before being sent to the World Vegetable Center headquarters in Taiwan. In Taiwan, a 2mm disk cut from each FTA card using a Harris micro-perforator was processed in a separate 1.5-ml micro-centrifuge tube according to the manufacturer's instruction (Whatman FTA® Card Technology). The purified bacterial DNA samples fixed on the discs cut from the FTA cards were amplified using the multiplex PCR protocol developed by Fegan and Prior (2005).

3.4 Species and Phylotypes identification: The species were determined using *R. solanacearum* species complex (RSSC) specific primer pairs AU759f and AU760r (Opina *et al.,* 1997) (Table 2). Two mm diameter of 74 FTA™ card containing DNA of each of the isolates were washed and dried, (Bihon *et al.,* 2020) and used for PCR amplification. A PCR reaction consisting of one-disc equivalent to 1.0 µL, 2.5 µL of 10X PCR buffer with 15 mM $MgCl_2$, 0.5 µL of 2.5 mM dNTPs, 1 µL of 10 µM of each primers, 0.2 µL of 5 U/µL Taq DNA polymerase and filled with 18.8 µL of sterile deionized water for a total volume of 25 µL reaction mixture. Amplification were conducted in a Bio-Rad DNA Engine ® Peltier Thermal Cycler with an initial cycle of 94 °C for 3 min, 53 °C for 1 min and 72 °C for 1.5 min, followed by 30 cycles of 94 °C for 18 s, 60 °C for 18 s and 72 °C for 18 s, with a final extension step of 72 °C for 5 min and holding at 4 °C. Phylotype identification were conducted using a multiplex PCR combining four phylotype specific primer (Table 2) and RSSC specific primer pairs. PCR amplification and reactions were conducted as described by (Bihon *et al.,* 2020). Each PCR assay included positive and negative controls. The amplified products were separated in 1.5% agarose gels in a 1×TXE buffer, stained with ethidium bromide and viewed under UV light for documentation. A 100 bp DNA ladder was used to estimate the size of the amplified products.

Table 2: Expected Amplicon sizes are mixed with Remark
List of primers used for the species specific (Opina *et al.*, 1997) and phylotype analysis (Fegan and Prior 2005).

Primer name	Primer sequence	Expected amplicon size (bp)	Remark
Nmult21 : 1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144	Phylotype I (*R. pseudosolanacearum*)
Nmult21 : 2F	5'-AAGTTATGGACGGTGGAAGTC-3'	372	Phylotype II (*R. solanacearum*)
Nmult22 : InF	5'-ATTGCCAAGACGAGAGAAGTA-3'	213	Phylotype IV (*R. syzygii*)
Nmult23 : AF	5'-ATTACSAGAGCAATCGAAAGATT-3'	91	Phylotype III (*R. pseudosolanacearum*)
Nmult22 : RR	5'-TCGCTTGACCCTATAACGAGTA-3'		Phylotype universal reverse
759R	5'-GTCGCCGTCAACTCACTTTCC-3'	280	Universal *RSSC*
760F	5'-GTCGCCGTCAGCAATGCGGAATCG-3'		

4 RESULTS

4.1 Survey and sample collection A total of 81 positive *R. solanacearum* strains using primers 759/760 wilted African eggplant (*Solanum aethiopicum)* and *Solanum melongena* in the three regions were characterized. Bacterial wilt is widely distributed in Guinea (Figure 1) but the majority of the positive samples (58, 2%) were collected in lower Guinea (Boké, Coayh, Kindia, Forékariah); the vegetable cropping area because of the proximity with capital city Conakry. Followed by Forest Guinea (Macenta, N'Zérékoré) 23, 6% and Upper Guinea (Kankan, Siguiri, Dabola) 18, 2% (Table3).

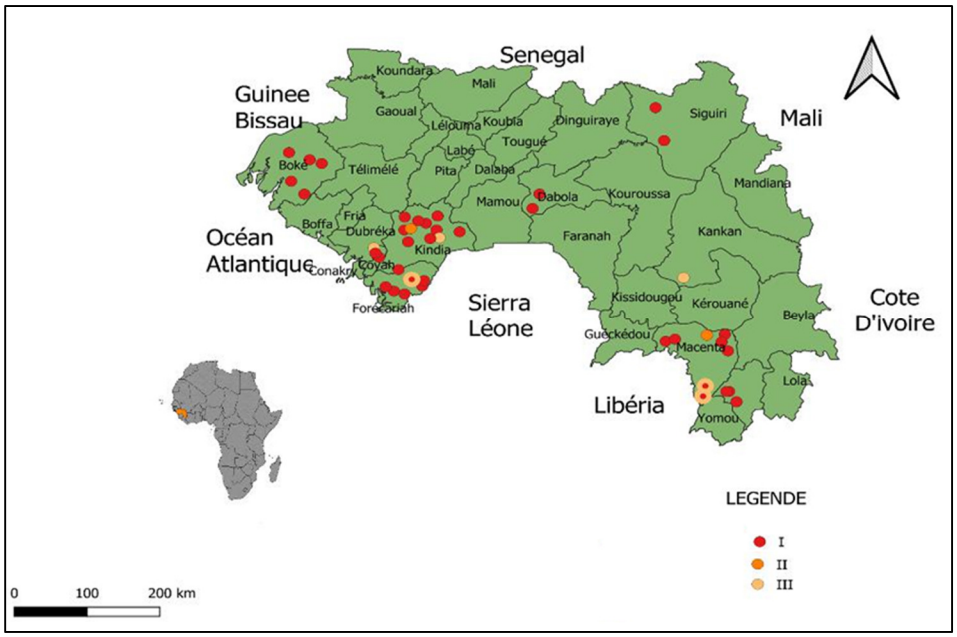

Figure 1; Geographic distribution of Strains phylotypes collected in Guinea

Table 3: Phylotypes distribution of Guinean strains within agroecological zones

Regions (AEZ)	Phylotype I	Phylotype II	Phylotype III	Total
Lower Guine	27 (49,1%)[a]	1 (1,8%)	4 (7,3%)	32 (58,2%)
Uper Guinea	9 (16,4%)	0	1 (1,8%)	10 (18,2%)
Forest Guinea	11 (20%)	1 (1,8%)	1 (1,8%)	13 (23,6%)
Total	47 (85,5%)	2 (3,6%)	6 (10,9%)	55 (100%)

[a]: Percentage of the different phylotypes within each agroecological zones (AEZ)

4.2 Phylotype Analysis Multiplex PCR for the identification of phylotypes, (Fegan and Prior 2005) revealed that despite the positivity of the 81 strains characterized as RSSC, 68% (n = 55) were subsequently assigned to a group of phylotypes (Table 3). And 85.5% of these 55 strains were phylotype I, 3.6% phylotype II isolates, 10; 9% phylotype III. The phylotypes of the remaining 26 RSSCs have not been identified, possibly due to the quality of the cards sent (Table 4).

Table 4: Proportion of samples in each collection by district, number of samples BW positive and Phylotypes identified

Prefectures (Districts)	Number of tested RSCC by primers 759/760	Number of Phylotypes determined	Percentage Phylotype
Boké	5	5	100
Coyah	2	1	50
Kindia	25	18	72
Forekariah	12	8	66.7
Dabola	4	4	100
Macenta	16	9	56.3
N'Zérékore	7	4	57.1
Kankan	1	1	100
Siguiri	9	5	55.,6
Total	**81**	**55**	**67.9**

5 DISCUSSION

This phylogenetic study on Eggplant shows that the prevalence of *R. solanacearum* 119 population in Guinea has considerable genetic diversity and that their composition and 102 distribution is highly specific when compared to other African situations, particularly in 121 Côte d'Ivoire, in Benin and Burkina Faso (N'Guessan *et al.,* 2012; Troaré O. 2019; 122 Sikirou *et al.,* 2015). Guinean strains identified on eggplant only were distributed in three 123 of the four phylotypes described: I, II, and III, as expected from previous reports in 124 Africa (Elphinstone 2005; Ravelomanatsoa *et al.,* 2018). 125 Phylotype I is the major group (82% of the collection), and it is widespread in the three 126 agroecological zones surveyed in Guinea. As in Benin, the survey on Gboma (*Solanuum macrocarpon*) classified all strains in *R. solanacearum* in Phylotype I, (Sikirou *et al.,* 2015) In Côte d'Ivoire, in the study of the Diversity of Ralstonia on Solanaceae, only four phylotypes were collected from eggplant species; one strain of phylotype I-44 and the three strains of phylotypes III (N'Guessan *et al.,* 2012). In Burkina Faso, 42 of 102 strains (41%) were collected from eggplant across the country and they were all phylotype I. (Troaré O., 2019) This study will serve as a starting point for a more in-depth study of genetic diversity on several solanaceae which is the starting point for the creation of resistant or tolerant varieties for the benefit of Guinean producers.

6 CONCLUSION AND APPLICATION OF RESULTS

Eggplants with bacterial wilt can be found in all regions of the Republic of Guinea, but unsurprisingly the incidence is greatest in the areas where eggplant is more intensively cultivated and has been commercially cultivated for a long period alongside other solanaceous crops; these tend to be the lower altitude regions with greater average temperatures and higher rainfall. *R. pseudosolanacearum* phylotype I is the predominant strain across the whole country in eggplants, and since phylotype I strains are reported to have originated in Asia, this may mean that there has been insufficient time since the introduction of these strains into West African for the local eggplant species/ cultivars / landraces to have been selected for tolerance or resistance. Further work is required to determine the relative susceptibility of West African eggplant species/cultivars/land races to the different phylotypes/races of the RSSC. The phylotype II strains detected in the two samples presenting apparent mixed infection of phylotype I and phylotype II may represent carryover of *R. solanacearum* phylotype II strains

introduced into Guinea in infected potato (*Solanum tuberosum*) setts from Europe (Gbonamou *et al.,* 2020). Again, more work is required to determine if these are originally potato strain(s) and how similar they are to the phylotype II Guinea potato strain (RTG2/RUN378) identified by Prior and Fagan, (2005). The observation that the phylotype II strains were only ever observed in mixed infection with phylotype I strains may mean they are only weakly pathogenic on eggplant and require the help of phylotype I strains to establish infection. The World Vegetable Center has identified several new *S. melongena* lines which, like the widely promoted EG203, show good resistance/tolerance to Asian phylotype I RSSC strains (and root knot nematode tolerance; RKN) and are potentially useful as resistant rootstock for grafting with tomato scions in Asia where BW and/or RKN are a problem. They may also have potential as rootstocks and fruit characteristics of their own which are suitable for the African market (Nordey et al.,2020), but before they are promoted more widely in West Africa they should be screened against several of the West African RSSC phylotype I and phylotype III strains both singly and as mixtures to assess if the resistance/tolerance to BW is likely to hold good and be durable. With climate change, the BW situation in West Africa is only likely to get worse, so that as well as screening for genetic resistance to BW, other approaches such as combining partial resistance with biological control (Subedi *et al.,* 2020) including perhaps the use of lytic bacteriophages (Álvarez *et al.,* 2019) against *R. pseudosolanacearum* should be explored. This study serves as a starting point for these more in-depth studies of the genetic diversity of RSSC strains in Guinea, the screening and breeding for resistance or tolerance against them and the search for local strains of biological control agents against them.

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Conflict of Interest: The authors of this work declare on their honour that they have no conflicts of interest.

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