Morphological and biochemical characterization of strains of *Ralstoniasolanacearum*, causal agent of bacterial wilt in tomatoes in Cameroon and screening of virulent strains.

ABSTRACT

*Ralstoniasolanacearum*is a major constraint in tomato production. The aim of this work was to identify the different biovars and races of *R. solanacearum* which infect tomato plants in Cameroon and determine the most virulent in a gnotobiotic environment. Thirty (30) samples were collected in the field of 03 different agro-ecological zones considered as major tomato production area in Cameroon. The bacteria were isolated on modified Kelman solid medium, and identified based on morphological and biochemical characterization. The pathogenicity test was performed in a gnotobiotic environment by root inoculation. Twenty-nine (29) isolates showed colonies characteristic of virulent strains. Water, motility, catalase, KOH, Kovac oxidase, glucose reduction, and sucrose tests were positive. However, gram staining, spore production, arginine test and sulfate reduction were negative. The biovar tests carried out revealed the presence16.66% biovar 2 (bv 2) and 76.66% biovar 3(bv 3). The tobacco hypersensitivity tests carried out revealed the presence of two (02) races: race 1 and race 3 (majority). A predominance of race3 biovar 3 was observed in Cameroon. Results from pathogenicity test revealed that two (02) strains, FM6 (race 3 bv2) and BFo (race 3 bv 3) were high virulent, causing up to 100% loss of seedlings on the sixth day post-inoculation.

Key words: *R. solanacearum*, tomato, biochemical characterization, virulence.

1.INTRODUCTION

Ralstoniasolanacearum, the causative agent of bacterial wilt is a serious challenge for various crop production worldwide. This bacterium is listed as the second most destructive plant pathogen in the world [1], and is responsible for massive losses in several commercially important crops such as tomato, chili pepper, eggplant, potato and legumes [2]. It infects in a wide range of climates including temperate, Mediterranean, subtropical and tropical climates where it is able of infecting nearly 450 plant species in 54 botanical families; both monocotyledons and dicotyledons[3].

Bacterial wilt of nightshade crops is present in many African countriesincluding Burundi, South Africa, Egypt, Libya, Nigeria, Zambia and Cameroon [4]. On tomatoes, *R.solanacearum* can cause losses of up to 91% in the field[5; 6].

In Cameroon, bacterial wilt constitutes a major constraint of the cultivation of nightshades crops. This disease has been reported in four of the fiveagro-ecological zones, namely the high Guinean savannah, the western highland, monomodal and bimodal rainfall forest. Three of the four phylotypes of *R.solanacearum* have been identified in Cameroon: the Asian phylotype I, the American phylotype II and the African phylotype III[6]. The classification system by races and biovars is currently recommended internationally [7; 8; 9]. Breeds were classified based on host range [7]. *Ralstoniasolanacearum* was grouped into five biovars based on the utilization or oxidation of three hexoses (mannitol, dulcitol, and sorbitol) and three disaccharides (lactose, maltoseandcellobiose) [8; 10]. Biovar 1 does not oxidize any disaccharide sugars and hexose alcohols;biovar 2 only oxidizes disaccharide sugars. Later, biovar 2 strains were divided into 2T and N2 based on their ability to utilize ribose and trehalose [11], biovar 2T strains have been isolated from potato, tomato, and eucalyptus and is found in three of the four phylotypes (phylotypes II, III, and IV) of *Ralstoniasolanacearum* [12], Biovar 3 oxidizes both disaccharide sugars and hexose alcohols, biovar 4 oxidizes only hexose alcohols while biovar 5 oxidizes sugar disaccharides and oxidizes only hexose alcohol mannitol but not sorbitol and dulcitol[8; 10].

The disease is difficult to control because *R. solanacearum* presents a very high phenotypic, genotypic and pathogenicity variability as evidenced by its wide host range, its plasticity and its ability to adapt to different agro-climatic environments[13]. Therefore, the characterization of *R. solanacearum* strains is important in developing effective strategies for diagnosis, quarantine and selection of effective control method [14]. The study of the infection characteristics of tomato cultivars of the three bacteria populations (biovar 1, biovar 2, biovar 3) showed that biovars have different behavior depending on temperature, cultivar and soil type[15].In Cameroon, the genetic diversity and phylogeny of the selected strains were evaluated by multiplex-polymerase chain reaction and the race-specific 3/biovar 2 PCR test [6]. This work revealed the presence of race 3/biovar 2 which corresponds to phylotype II. However, phylotype II strains represented 35% of the collection and among them, 5% were identified as sequevar 1 (R3bv2) because the characteristic 304-bp band specific to R3bv2 was amplified from their DNA using primer pair 630/631**.** Furthermore, it finds the races and biovars in the 03 phylotypes identified in Cameroon, therefore the

information on these races and these biovars which infect nightshades in general and tomatoes in particular in Cameroon is incomplete. Likewise, information on virulent strains is not known.

Tomato plants, as well as *Arabidopsis thaliana*, *Phaseolus vulgaris* (distant host plant) are model plants that have been used to understand the virulence, host resistance and evolution of *R. solanacearum*, inoculated either by wetting the soil, or by inoculating the stems[16; 17]. These two methods require adult tomato plants approximately 45 days old for the study of infection. It takes at least 60 days to complete an infection study using either of these two strategies. Additionally, as seedlings are grown in pots containing soil, the seedlings associated with soil microbiota, including other bacteria which cannot be avoided. This soil microbiota may interfere with the infection process as well as subsequent observations, meaning that disease patterns may not be due to *R. solanacearum* inoculation alone[18].Growing and maintaining a large number of adult plants requires a lot of space and time as well as economical investments[19].

To deal with this challenge, recent work provided an innovative method which consisted of inoculating the roots of 6 to 7 days-old tomato plant with *R. solanacearum*in a gnotobiotic environment, which is equally effective in causing diseases in different tomato cultivars [19; 20], especially since *R. solanacearum* naturally infects the host plant through the roots. This method is acceptable, fast, effective, less expensive and requires less bacterial inoculum.

The present study was initiated to determine the different biovars and races of *R. solanacearum* which infect tomato plants in Cameroon and to determine the most virulent in a gnotobiotic environment.

2. MATERIAL AND METHODS

2.1 Material

Tomato plants showing the symptoms of the disease, a cooler, plastic and glass Petri dishes 90 mm in diameter, beakers, cotton, 70° ethyl alcohol and bleach were used.

2.2 Methods

2.2.1Sample collection

Thirty (30) samples of diseased tomato plants were taken in October and November 2020 from farmer's plantations in different localities of three agro-ecological zones of Cameroon where tomatoes are widely cultivated.Fourteen (14) samples were collected in the Western highlands (inBandjoung, Baham, Mbouda, Foumbot, Dschang, Bafou), twelve (12) samples in the forest zone with bimodal rainfall (in Bafia, Ossimbi, Wasse, Obala, Nkometou, Yaounde, Metet) and four samples (04) in the forest zonewithmonomodal rainfall (in Melong and Njombe).Sampling was done randomly in each plot containing infected tomato plants, each infected plant being collected entirely with its roots in the field. Disease symptoms were studied by visual observation according to the standard procedure [21]. The plants thus harvested were each wrapped in plastic and stored in a refrigerator then sent to the laboratory for bacteria isolation.

2.2.2Designation of the isolates collected

The isolates collected were designated according to the sampling area [22]. In the Western Highlands, isolates BJ1 and BJ2 mean that these isolates were collected in Bandjoung in site 1 and 2 respectively; MB1 and MB2 taken from different Mbouda sites; BH1 and BH2 in Baham; FM1, FM2, FM4, FM5, FM6 in Foumbot; DscforDschang; BFO1 and BFO4 to Bafou.In the forest zone with bimodal rainfall, the OB1 isolate collected in obala; NK1, NK2, NK3 and NK3' taken from different sites in Nkometou, W1 and W2 from different sites in Wassé (Ombessa); OSS in Ombessa center; BF0, BF2 in different sites in the Bafia area; Ydé1 and Ydé5 in different sites in Yaoundé. In the forest zone with monomodal rainfall, the NKG1, NKG2, NKG2' isolates were taken from different sites in the Nkongsamba (Melong) zone, LTn on the coast precisely in Ndjombe.

2.2.3Observation of visual symptoms (water test)

A very simple method was used to make a diagnosis of *R. solanacearum*. It consists of taking a fragment of stem from the lower levels, disinfecting it superficially with 95% ethyl alcohol for one minute then rinsing it 5 times with sterile distilled water and finally immersing it in a glass of water. After 5 minutes, white ooze made of billions bacteria appeared showing the presence of the disease[23; 24; 25].

2.2.4 Isolation of *Ralstoniasolanacearum*

Ralstoniasolanacearum is easily isolated on agar media, in particular on Kelman's medium [26] supplemented with tetrazolium chloride (TTC). The stem of each infected plant sample was sanitized respectively with 70% alcohol and 2% bleach then rinsed with sterile

distilled water. The disinfected stem of each sample was cut approximately 10 cm in length then split into two half-cylinders.The section part of each half-cylinder of the rod was deposited directly in contact on Kelman media enriched with tetrazolium chloride [26] contained in the Petri dishes. One liter of modified Kelman medium contains: 10.0 g of peptone, 1.0 g of casamino acid (Difco), 17g of agar, 5 ml of glycerol, then added with 5 ml of 1% tetrazolium chloride (TTC).Different bacterial colonies which grew on Kelman medium and showing a morphological appearance characteristic of *R. solanacearum* were purified by exhaustion method on solid medium. Then the boxes thus treated were inverted and incubated in an oven at 28°C for 48 hours.

2.2.5 Macroscopic characteristic

The macroscopic observation was done to describe the morphological features of the bacterial colonies. It was done either with the naked eye or with a magnifying glass. During this analysis, the aspect, the consistency, shape and color of the bacterial colonies were determined, as well as the presence or absence of spores.

2.2.6Motility test

To confirm the presence or absence of flagella in the bacteria studied, the motility test was carried out. It consists of placing a drop of the bacterial suspension of $3x10^8$ CFU/ml (0.1 McFarland) on a slide covered with an object cover sheet. Slides were observed under a microscope at magnification 60X[27].

2.2.7Biochemical tests

Gram stain

Young pure cultures (24 hours) were used for Gram staining. A loop of the bacteria was spread on a glass slide and fixed by heating over a very weak flame. An aqueous solution of crystal violet (0.5%) was spread on the smear for 30 seconds and then washed with running water for one minute. It was then flooded with iodine for one minute, rinsed in water tape, and decolorized with 95% ethanol until colorless flow[28].

KOH test

Potassium hydroxide KOH solution (3%), a sterile microscope slide and toothpick were used to complete the Gram test on isolated bacterial colonies.

*Ralstoniasolanacearum*isolates were collected aseptically from 24- to 36-hour-old culture plates using a sterile toothpick. They were then placed on a glass slide in a drop of 3% KOH solution, shaken for 10 seconds and observed for the formation of slime threads[28].

Catalase test

Young cultures of *R. solanacearum* (18 to 24 hours) and 3% hydrogen peroxide (H_2O_2) were used to observe the production of gas bubbles. A full loop of bacterial culture was mixed with a drop of H_2O_2on a glass slide and observed for gas bubble production by eye and under 25X dissection magnification [29]. The formation of bubbles corresponds to catalaseproduction classifying the bacteria as catalase-positive bacteria (catalase +) while the non-production of bubbles corresponds to the absence of catalase and consequently catalase-negative bacteria (catalase-).

Kovac oxidase test

The oxidase test identifies bacteria that possess the enzyme cytochrome oxidase. N,N-Dimethyl-phenylene diamine Dihydrochloride 1% (100 ml) was prepared and stored in a dark rubber stopper bottle. A drop of reagent was added to a piece of filter paper (Whatman no. 1) placed in a glass Petri dish. Small amounts of inoculum were rubbed onto the filter paper impregnated with 1% (w/v) oxidase reagent solution. The bacteria were then noted for development of purple color within 10 to 60 seconds indicating oxidation of the reagent[30].

Arginine test

For arginine dihydrolase activity in Thornley's medium contained in a test tube, the medium was inoculated with a loop of *R. solanacearum* colonies and then incubated for 24 hours at 28ºC. The positive reaction for the degradation of arginine to ornithine + CO2 + NH3 was verified with a color change from mid-purple to yellow[29].

TSI test (three sugars and iron)

One liter of TSI medium containing 3 g of meat extract, 3 g of yeast extract, 20 g of peptone, 5 g of sodium chloride, 10 g of lactose, 10 g of sucrose, 1 g of glucose, 300 mg of ferrous ammonia sulfate, 24 mg of phenol red, 300 mg of anhydrous sodium thiosulfate and 11 g of agarwasprepared.The TSI test was performed based on the ability or inability of bacteria to ferment glucose (with or without the release of gas), lactose, sucrose and to reduce sulfates to sulfides which, in the presence of iron, gives a black precipitate of iron sulfide. The young

cultures of *R. solanacearum* (24 hours) were inoculated on the slope of the TSI medium by streaks and in the base by puncture of a Pasteur pipette previously flame sterilized, then left to incubate for 24 hours at a temperature of 37°C[27].

Biovar Determination Test

The technique used to distinguish the different biovars of *Ralstoniasolanacearum* within the different isolates was based on biochemical tests relating to the abilities of the strains to use and/or to oxidize three disaccharides, in particular maltose, lactose and cellobiose as well as three hexose alcohols mannitol, sorbitol and dulcitol. Biovar 2 biotypes was recognized according to their use of trehalose and/or meso-inositol[8; 31].The basic medium used for this purpose consisted of 1.0 g Bacto-peptone, 1.0 g $NH_4H_2PO_4$ (Ammonium dihydrogen phosphate), 0.2 g KCl (Potassium chloride), 0.2 g of $MgSO_4$, $7H_2O$ (magnesium sulfate), 0.03 g of Bromothymol Blue, 3.0 g of Agar in one liter of distilled water 1,000 ml[8 et 32].The pH was adjusted between 7 and 7.1 by adding a few droplets of 40% weight/volume NaOH solution, then the medium was mixed by heating and constant stirring. The medium thus obtained had an olive-green color. It was distributed in beakers in 90 ml aliquots corresponding to the number of carbohydrates to be tested before autoclaving at 121°C for 21 minutes. All sugar solutions were sterilized by filtration (Seitz or 0.22-micron millipore membrane) in previously sterilized test tubes. Approximately 10 ml of each 10% carbohydrate solution was added to 90 ml of the basic medium cooled to 60°C in order to obtain a final concentration of 1%.The solution was distributed using a precision pipette, i.e. 200 µl into each of the 96 sterile wells of culture dishes, keeping one row per sugar. The inoculum was a 48-hour bacterial culture. The wells containing the different sugars were inoculated by taking colonies of pure strains using sterile Pipettes. Three repetitions per sugar (on the same line) and per strain was done. Negative controls were not containing bacteria.

2.2.8Tobacco hypersensitivity test

The tobacco hypersensitivity test is a test developed by [33]. It was used to distinguish between race 1 (which infect tobacco, tomato, chili pepper, pepper, eggplant, ornamental diploid bananas, legumes, Euphorbiaceae, Casuarinaceae and Rubiaceae), race 2 (which infect triploid bananas and some diploids and other Heliconiaspp) and race 3 (which infects potatoes and tomatoes). This test was carried out using tobacco leaves by infiltration of bacterial cells into the leaf parenchyma with a syringe. Wilting and death of tobacco leaves after eight (8)

days indicates race 1. Yellowing of the infiltration area indicated race 3. Hypersensitivity with white necrotic tissue in the area between the veins indicated race2.

2.2.9Screening of virulent strains

The study of the virulence of the strains was carried out under gnotobiotic conditions using the method of[19].

Germination of tomato plants for inoculation

Tomato seeds of the Roma vf variety, very susceptible to bacterial wilt, were pre-soaked in sterile distilled water for two days. They were then dispersed on sterile moist tissue paper in a plastic tray in a growth chamber (Orbitek) maintained at a temperature (T) of 28°C, 75% relative humidity (RH) and a photoperiod (L/D) of 12 hours (Figure 1a).TheWatering were done after every two days with sterile distilled water. Seven (07) days after germination of the seeds, the seedlings were obtained on the bed of tissue paper[18].

Preparation of bacterial inoculum

Ralstoniasolanacearum was subcultured on modified Kelman medium, then left to incubate for 24 hours at 28°C. The 24-hour-old colonies were suspended in sterile distilled water, then calibrated at 10^8 CFU/ml using a spectrophotometer at a wavelength corresponding to 600 nm and an optical densityO. D= 0,1 [34].

Root inoculation of *R. solanacearum* in tomato plants

Approximately 15 to 20 ml of inoculum of each *R. solanacearum* strain (approximately 10^8 CFU/ml) was collected in a sterile container. The 6 to 7 day-old tomato seedlings mentioned were each immersed in the bacterial inoculum (up to the root-shoot junction) (figure 1c). They were subsequently transferred into an empty sterile microcentrifuge tube of 1.5 to 2 ml (figure 1d), tube to which 1 to 1.5 ml of sterile water were added (figure 1e). Forty (40) plantlets were used for each bacterial inoculum. As controls, forty (40) seedlings were also inoculated with sterile distilled water following the same procedure as previously.All inoculated seedlings as well as the controls were transferred to a growth chamber maintained at 28°C, 75% RH and L/D of 12 hours (Figure 1g). The following day, the seedlings were observed to assess the progression of the disease.

Determination of the rate of dead tomato seedlings

Dead tomato seedlings were counted daily. The rate of dead seedlings in the treated batch was determined in relation to the total number of seedlings inoculated.

2.2.10 Statistical analysis

An ANOVA comparison of variance followed by a Turkey's multiple comparison tests was then performed. The level of significance was assessed at threshold 5%.

3.RESULTS AND DISCUSSION

3.1 Results

3.1.1Morphological and biochemical characterization of *Ralstoniasolanacearum*

The results showed that all bacterial isolates were recovered on Kelman culture medium; the colonies were clearly visible and remarkable. Of all the isolates (30), twenty-nine (29) presented colonies with a mucous appearance, irregular in shape, whitish in color evolving to pink in the center (Figure 2a). On the other hand, isolate MB1 presented colonies with rough shapes, with small round colonies of bright red color (figure 2b). Spore formation was not observed in all isolates.

All cut stems from symptomatic plants harvested and immersed in water exhibited a whitish ooze in clear water. Table 1 and Figure 2 show that all isolates tested were positive for motility, catalase, oxidase and KOH tests. But a negative reaction was observed for Gram stain and arginine test.

Table 1.Microscopic observations and biochemical tests

agro-ecologicalsampling zones	isolates codes	motility test	Gramstains	KOH test	catalase test	oxydasekovac test	arginine test
	BJ1	+	-	+	+	+	-
	BJ2	+	-	+	+	+	-
	BH1	+	-	+	+	+	-
	MB1	+	-	+	+	+	-
	MB2	+	-	+	+	+	-
Western Highlands	DSC	+	-	+	+	+	-
	BFO4	+	-	+	+	+	-
	FM1	+	-	+	+	+	-
	FM2	+	-	+	+	+	-
	BH2	+	-	+	+	+	-
	FM4	+	-	+	+	+	-
	FM5	+	-	+	+	+	-
	FM6	+	-	+	+	+	-
	BFO1	+	-	+	+	+	-
	W1	+	-	+	+	+	-
	W2	+	-	+	+	+	-
	BF_0	+	-	+	+	+	-
Forest zone with bimodal rainfall	BF2	+	-	+	+	+	-
	OSS	+	-	+	+	+	-
	OB1	+	-	+	+	+	-
	NK1	+	-	+	+	+	-
	NK3	+	-	+	+	+	-
	NK3’	+	-	+	+	+	-
	Ydé1	+	-	+	+	+	-
	Ydé5	+	-	+	+	+	-
	Met	+	-	+	+	+	-
Forest zone with monomodal rainfall	NKG1	+	-	+	+	+	-
	NKG2	+	-	+	+	+	-
	NKG2’	+	-	+	+	+	-
	LTn	+	-	+	+	+	-

Furthermore, the TSI test (table 2, figure 2f) revealed that all the isolates fermented glucose (yellow pellet showing: positive glucose) with the release of gas in sixteen (16) isolates. Similarly, all isolates fermented lactose and sucrose, but no reduction in sulfate was observed.

Table 2. TSI test

Agro-ecologicalsampling zones	isolates codes	lactose and/or sucrose slope	cheek	gas	H_2S
	BJ1	+	+	-	-
	BJ2	+	+	-	-
	BH1	+	+	-	-
	MB1	+	+	-	-
	MB2	+	+	+	-
Western Highlands	DSC	+	+	+	-
	BFO4	+	+	-	-
	FM1	+	+	+	-
	FM2	+	+	-	-
	BH2	+	+	+	-
	FM4	+	+	+	-
	FM5	+	+	+	-
	FM6	+	+	-	-
	BFO1	+	+	-	-
	W1	+	+	+	-
	W2	+	+	+	-
	BF_0	+	+	-	-
	BF2	+	+	+	-
	OSS	+	+	+	-
Forest zone with bimodal rainfall	OB1	+	+	-	-
	NK1	+	+	-	-
	NK3	+	+	+	-
	NK3’	+	+	-	-
	Ydé1	+	+	-	-
	Ydé5	+	+	+	-
	Met	+	+	+	-
Forest zone with monomodal rainfall	NKG1	+	+	+	-
	NKG2	+	+	+	-
	NKG2’	+	+	+	-
	LTn	+	+	-	-

3.1.2 Biovar and tobaccohypersensitivity

Based on the biovar determination test, twenty-three (23) isolates oxidized both disaccharide sugars (cellobiose, lactose, maltose) and hexose alcohols (mannitol, dulcitol, sorbitol), and were identified as biovar 3 (Table 3). Oxidation is characterized by the change in color of the medium which turns from olive green to yellow (figure 2e).Five (05) isolates (FM1, FM6, BJ1, BJ2 and Yde 5) oxidized only the disaccharide sugars, characteristic of biovars 2; FM1, FM6 and BJ2 isolatesdid not oxidizedtrehalose and meso-inositol, therefore are of biotype N2.BJ1 and Yde 5 isolates, which oxidized these twosugars are of biotype2T. The biovars of the two (02) isolates MB1 and FM2, collected in the western highlands have not been determined. However, the control plates for the different sugars and sugar alcohols remained unchanged (figure 2e).

The results of tobacco leaf infiltration using the different isolates showed that FM2, FM4, OSS, OB1, NK3', Yde 5, NKG1, NKG2, NKG2' and LTn isolates caused wilting of tobacco leaves 5 days'post-inoculation, which indicates their belonging to race 1 (Table 3). However, the other isolates, except MB1isolate, caused leaves yellowing 72 hours after inoculation on tobacco leaves. Tobacco leaves infiltrated with sterile water and isolate MB1 showed no reaction.

Table 3. Biovar and breed determination tests

agro-ecologicalsampling zones	isolate codes	cellobiose	lactose	maltose	mannitol	dulcitol	sorbitol	tréhalose	mésoinositol	Biovars	Breed
	BJ1	+	+	+	-	-	-	+	+	2T	3
	BJ2	+	+	+	-	-	-	-	-	N2	3
	BH1	+	+	+	+	+	+			3	3
	MB1	+	-	+	-	-	-			ND	ND
Western Highlands	MB2	+	+	+	+	+	+			3	3
	DSC	+	+	+	+	+	+			3	3
	BFO4	+	+	+	+	+	+			3	3
	FM1	+	+	+	-	-	-	-	-	N2	3
	FM2	-	+	+	-	-	+			ND	1
	BH2	+	+	+	+	+	+			3	3
	FM4	+	+	+	+	+	+			3	1
	FM5	+	+	+	+	+	+			3	3
	FM6	+	+	+	-	-	-	-	-	N2	3
	BFO1	+	+	+	+	+	+			3	3
	W1	+	+	+	+	+	+			3	3
	W2	+	+	+	+	+	+			3	3
	BF_0	+	+	+	+	+	+			3	3
Forest zone with bimodal rainfall	BF2	+	+	+	+	+	+			3	3
	OSS	+	+	+	+	+	+			3	1
	OB1	+	+	+	+	+	+			3	1
	NK1	+	+	+	+	+	+			3	3
	NK3	+	+	+	+	+	+			3	3
	NK3'	+	+	+	-	+	+			3	1
	Ydé1	+	+	+	+	+	+			3	3
	Ydé5	+	+	+	-	-	-	+	+	2T	1
	Met	+	+	+	+	+	+			3	3
Forest zone with monomodal rainfall	NKG1	+	+	+	+	+	+			3	1
	NKG2	+	+	+	+	+	+			3	1
	NKG2'	+	+	+	+	+	+			3	1
	LTn	+	+	+	+	+	+			3	1

ND: not determined; + positive; - negative

3.1.3Screening of virulent strains

Pathogenicity tests in a gnotobiotic environment showed bacterial wilt symptoms within 48 hours after root inoculation (Table 4). Almost all strains caused the death of seedlings. A significant difference in the rate of seedling death was observed between non-inoculated

plants (control) and inoculated plants. Besides, the plants not inoculated with *R. solanacearum* did not show any symptoms of the disease.This result indicates that seedling death is caused by *R. solanacearum*. The FM6 and BFo strains were high virulent causing 100% dead of the inoculated seedlings sixdays' post-inoculation.

Table 4. Rate of dead seedlings after root inoculation using the different strains

agro-ecologicalsampling zones	strains	day 1	day 2	day 3	day 4	day 5	day 6	day 7
	FM1	0h	7 h	12 g	23 f	51 cde	70 bcd	80 bc
	FM2	0h	8 h	15 g	28 f	58 cde	75 bc	86 ab
	FM4	0 h	10 g	18 g	34 ef	61 bcd	80 bc	90 ab
	FM5	0 h	14 g	25 f	40 ef	66 bcd	85 ab	95 a
	FM6	0 h	25 f	50 def	78 bc	90 ab	100 a	100 a
Western Highlands	BFO1	0 h	4 h	8 h	18 g	40def	63bcd	71bc
	BFO4	0h	20 g	30 f	54 cde	70 bcd	88 ab	98 a
	DSC	0 h	12 g	25 f	50 def	78 bc	90 ab	100 a
	BJ1	0h	15 g	25 f	40 ef	65 bcd	85 ab	95 a
	BJ2	0h	4 h	12 g	25 f	55 cde	72 bc	85 ab
	BH1	0h	5 h	12 g	25 f	56 cde	73 bc	85 ab
	BH2	0 h	0 h	5 h	12 g	41 def	59 cde	70 bcd
	MB2	0h	2 h	5 h	15 g	44 def	62 bcd	72 bc
	W1	0 h	0 h	0 h	2 e	5 h	28 f	45def
	W2	0 h	0 h	1 h	5 e	10 g	32ef	50def
	OSS	0 h	4 h	8 h	17 g	46 def	65bcd	75bc
Forest zone with bimodal rainfall	BF0	0 h	26 f	51 cde	75 bc	91 ab	100a	100a
	BF2	0 h	17 g	38 ef	55 cde	65 bcd	79 bc	95a
	NK1	0 h	23 f	48 def	67 bcd	78 bc	95 a	100 a
	NK3	0 h	4 h	12 g	25 f	55 cde	72 bc	85 ab
	NK3'	0 h	10 g	18 g	35 ef	60 cde	80 bc	90 ab
	OB1	0 h	0 h	1 h	8 h	17 g	42 def	60 cde
	Ydé1	0 h	17 g	38 ef	55 cde	65 bcd	79 abc	95 a
	Ydé5	0 h	0h	5 h	12 g	41 def	59 cde	70 bcd
	Met	0 h	0h	0 h	2 h	5 h	28 f	45 def
Forest zone with monomodal rainfall	NKG1	0 h	0 h	1 h	5 h	12 g	30 f	46 def
	NKG2	0 h	0 h	2 h	7 h	8 h	40 ef	55 cde
	NKG2'	0 h	0 h	7 h	15 g	38 ef	60 cde	80 bc
	LTn	0 h	5 h	10 g	20 g	45 def	65 bcd	90 ab
	Witness	0 h	0h	0 h	0 h	0 h	0 h	0 h

The values followed by different letters inside the column are significantly different at the 5% threshold according to the Tukey'stest.

3.2 Discussion

The whitish ooze exuded into clear water from the stems of sampled tomato plants confirms the presence of *R. solanacearum*. Indeed, [35] showed that vascular tissue cut from the stem and placed in water exudes oozing threads bacterial. A similar detection of*R. solanacearum* was carried out by [36] in Bangladesh on stem fragments of Solanaceae plants.

All isolates obtained in this study were positive on Kelman medium, known as the widely used and best studied medium to characterize *R. solanacearum*[37].Thevirulent and non-virulent colonies were distinguished among the isolates grown on Kelman medium. Twenty-nine (29) isolates had colonies with a mucous appearance, an appearance due to the production of exopolysaccharides (EPS) as described by [26]. Their irregular shape and their whitish color with a pink center confirm that *R. solanacearum* species were pathogenic bacteria as suggested by[38].One (01) isolate was characterized by colonies with a rough appearance due to the accumulation of triphenylformazan, stable dark red characteristic of non-virulent strains as mentioned by [26]. Our results are similar to those obtained by [29] and [28] during the morphological characterization of *R. solanacearum* strains on solid media enriched with tetrazolium chloride. Similarly, [39] in his work distinguished virulent strains of *R. solanacearum* from non-virulent strains, which were bright red, round, small and dry.

Gram stain results revealed that*R. solanacearum* cells were Gram negative, straight rod shaped or curved, which characterizes plant pathogenic bacteria as mentioned by [40]. [41], [42] reported a similar morphological and coloring result of *R. Solanacearum*.Furthermore, the KOH test indicated that the bacteria were confirmed as Gram negative because it produced strands of viscous materials when treating the bacterial culture with 3% KOH on a glass slide as described by [43]. Such results were also obtained by [44] when they performed the KOH test on *R. solanacearum* strains isolated from nightshades in Chile. All Gram-negative bacteria isolatedproduced gas bubbles when mixed with a drop of 3% H_2O_2on a glass slide, thus confirming the presence of the facultative aerobic bacteria. Our findings also corroborate with those obtained by [45] when carrying H_2O_2test on *R. solanacearum* isolated from tomato plants in Egypt.The Kovacs oxidase test differentiated isolates tested with all

isolates producing a dark purple color within 15–30 seconds indicating that they were aerobic bacteria [30]. Furthermore, the arginine test was negative because of the inability of the bacteria to degrade arginine, results also reported by [46] and [47] during the biochemical characterization of *R. solanacearum* isolated from the eggplant, tomato and potato.

All isolates studied here were mobile and did not present spores, which confirms the fact that *R. solanacearum* has one or more polar flagella making it mobile. [48] demonstrated that the mobility significantly contributes to the pathogenicity of the bacteria at the initial stage of infection. All *R. solanacearum* isolates obtained from diseased tomato plants by [45] showed the same results.

TSI test revealed that the isolates had oxidized lactose, sucrose. [27] obtained similar results when characterizing *R. solanacearum* isolated from ginger plants. Likewise, the isolates oxidized glucose with release of gas, this result also corroborates with those of [49], [50] who reported that *R. solanacearum* was positive in gas production in the presence of glucose. However, the results concerning the negative production of gaseous hydrogen sulphide (H2S) corroborate with those of [51] and [36] who carried out the same test on *R. solanacearum*. All the above-mentioned results categorically proved that the tomato plant isolates were the pathogen *R. solanacearum*.

Two biovars (biovar 2 and biovar 3) have been identified as the cause of tomato infection in the three agro-ecological zones. The differentiation of *R. solanacearum*biovars based on carbohydrate utilization was also reported by [8], [52] who differentiated strains that oxidized only disaccharides (cellobiose, lactose, maltose), characteristic of biovar 2; and by [46] and [48] who differentiated strains which oxidized both disaccharides and sugar alcohols, characteristic of biovar 3, results similar to ours. The third category corresponds to biovars which have not been determined (02 isolates from the Western highlands).These results, unlike the study of [6] which only reported the presence of *R. solanacearum*biovar 2 in Cameroon; confirm the presence of biovar 2 and biovar 3 which infect tomato plants. Based on oxidation of trehalose and meso-inositol as described by Hayward [11], the 2T and N2 biotypes were also determined with dominance of the N2 biotype.The Bj1 and Yde5 isolates are of biotype 2T, results that corroborate those of [12] who reported that 2T biovar strains isolated from tomato in southwestern Iran are also responsible for tomato infection in tropical areas.Wide diversity was observed among isolates collected from the Western Highlands where all three groups were identified. The study of [53] and [50] revealed that the effect of

temperature on *R. solanacearum*growth depended on biovars. They showed that the biovar 2 strains preferred low temperatures (22°C) for their growth while the biovar 3 strains grew at higher temperatures (27 - 37°C). The thermal profile could justify the distribution of biovars in the different agro ecological zones.Furthermore [6] mentioned that in Cameroon, thebiovar 2 strains adapt to climates with relatively colder temperatures (case of the Western highlands) compared to biovar 3 strains, which grow well in higher temperatures (case of agro-ecological zones with bimodal and monomodal rainfall) in Cameroon.

The tobacco hypersensitivity test showed the presence of two races (02) which infect tomatoes in Cameroon (race 1 and race 3 which is predominant). *R. solanacearum* may have triggered a hypersensitivity reaction in tobacco leaves. Indeed, this bacterium has an HR III type secretion system (T3SS), this secretion mechanism plays a role in the pathogenesis process which can trigger a hypersensitivity reaction[36; 47]. All isolates in the areaforest with monomodal rainfall were of race 1, it is present on all continents, except Europe [54; 55]. On the other hand, race 3 is the most represented in our collection and most present in the three agro-ecological zones. The work of [56] showed that unlike race 1 which has its optimum growth at high temperatures (around 35°C), race 3 establishes itself rather in temperate environments and has optimal development around 27°C.

Four groups of *R. solanacearum* infect tomatoes in Cameroon: race 1 biovar2, race 1 biovar3, race 3 biovar2 and race 3 biovar3. Of the five (05) biovars 2 identified, four (04) were isolated in the western highlands and 3/4 corresponded to race 3 biovar2 adapted to cold areas.This result corroborates with those obtained by [6] who isolated race 3 biovar 2 strains on tomato plants collected from the Western Highlands, a mountain growing area with a temperate climate. Races 1 biovar3 and races 3 biovar3 predominate in hot and humid zones (agro-ecological zones with monomodal and bimodal rainfall).In Africa, race 1biovar2 had also been isolated from tomato plants in Egypt by [45], race 1 biovar 3 in Benin by [9], race 3biovar 2 in South Africa by [14], race 3 biovar 3 in Mali by[56].

The extensive pathogenicity of*R. solanacearum* in 6-7day old tomato plants within 48 hours of root inoculation are evidenceofits virulence in the very early stages of the plant. Furthermore, the curvature, characteristic of the upper region of the shoot at the beginning of the disease observed in the seedlings after inoculation by the roots, indicates that the death of the seedlings is due to *R. solanacearum*[19].The authors [57] who studied the functions of virulence in a gnotobiotic environment demonstrated using gus staining and fluorescence

microscopy that wilted tomato seedlings were colonized by *R. solanacearum*.The strains FM6 (race 3 biovar) and BFo (race 3 biovar 3) were found to be the most virulent. This virulence known to be due to the expression of *R. solanacearum* virulence genes such as hrpB, phcA and pilT [58]. [18] Obtained similar results when they carried out pathogenicity tests by root inoculation of tomato seedlings in a gnotobiotic environment.The work of [59]has shown that the auxin secreted by *R.solanacearum* is involved in the early stages of infection in tomatoes and accentuates the symptoms of the disease; they have demonstrated that several auxin signaling and transport mutants induce tomato resistance to bacterial wilt. In addition, the work of [60] reported that during in vitro inoculation of *R.solanacearum*, effector proteins (T3SS and T3E) called type III effectors, whose expression is regulated by the bacterial protein HrpB, are the main determinants of the pathogenicity of the bacterium responsible for the various morphological alterations and the blocking of the growth of the primary roots of host plants with induction of disease symptoms.

4. Conclusion

This study revealed the presence of two races (1 and 2) and two biovars (2 and 3) which infect tomatoes in Cameroon. All the 04 isolates from the agro-ecological zone with monomodal rainfall are strains of race 1 biovar 3.However, almost all the strains in the agro-ecological zone with bimodal rainfall are race 3 biovar 3. All the diversity of strains found in Cameroon occurred in the western highlands. Race 3 biovar 3 (BFo) and race 3 biovar 2 were more virulent.

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CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

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