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Distribution, pathological and biochemical characterization of *Ralstonia solanacearum* in Benin

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ABSTRACT

In 2006 and 2007, 75 strains of *Ralstonia solanacearum* were collected from wilting tomato, pepper and eggplant in Benin. The distribution and the incidence of tomato bacterial wilt in the field were assessed by counting wilted tomato plants on 3 plots of 50 m^2 per field. The isolated bacterial strains, including the reference strain, were identified using ELISA, pathogenicity test and carbohydrate oxidation. Bacterial wilt is widely distributed in Benin and was found in five of the eight agro-ecological zones (AEZ) of Benin, which correspond to eight of the 12 districts of Benin. The disease was more severe in ferralitic soil (AEZ V), in valleys and lowlands (AEZ IV) and in highlands (AEZ I). The incidence of tomato bacterial wilt was up to 71%. No *R. solanacearum* strains were isolated from AEZ II, AEZ VII and AEZ VIII. Strains identified as *R. solanacearum* were more widely distributed in the south than the center and the north of Benin. Based on biochemical characteristics, Beninese *R. solanacearum* strains were grouped into biovar I/race1 and biovar III/race 1.

Introduction

Tomato (*Solanum lycopersicon*) is one of the most widely grown vegetable crops in the word (FAO, 1988). In Benin it is the most cultivated vegetable crop and important to the livelihoods of many people in *peri*-urban and rural areas (Agossou et al., 2001). It is used fresh and also processed as paste, juice, powder, or whole, providing a significant dietary source of vitamin A and C. Bacterial wilt, caused by the soil borne bacterium *Ralstonia solanacearum*, is regarded as a major limiting factor for tomato production in Benin, with yield losses of 100% in some area (Sikirou et al., 2001, 2009). Lebeau et al. (2011) reported that bacterial wilt inflicts severe economy losses in many crops worldwide.

Symptoms of *R. solanacearum* on tomato include wilting and necrosis as well as vascular browning (Swanson et al., 2005). Typically, stem and tuber cross-sections ooze whitish bacterial exudates (Genin and Boucher, 2002). The bacterium survives in infected plants, volunteer crops, susceptible weed hosts and infested soil (Hayward, 1994). It is disseminated mainly through use of infected plants, latently infected planting material, and contaminated irrigation water (Hayward, 1994).

The bacterium is widely distributed in tropical, subtropical and temperate regions worldwide on a range of host plants including food crops, cash crops, vegetables and fruits crops (Ji et al., 2005). Bacterial wilt of tomato has been reported in America (Denny and Hayward, 2001; Kim et al., 2003), Europe (Van Elsas et al., 2001), Asia (Fegan, 2005; Elphinstone, 2005) and Africa.

Strains of *R. solanacearum* are diverse in host range, pathogenicity, biochemical and physiological properties, geographical distribution, and epidemiological relationships (Poussier et al., 1999; Horita and Tsuchiya, 2001). The species was subdivided into six races according to host range (Buddenhagen and Kelman, 1964; Pegg and moffett, 1971) and into five biovars (Hayward, 1964; Hayward et al., 1990) based on carbon source utilization. There are numerous subtypes within the biovars that may be associated with particular geographical locations (Buddenhagen and Kelman, 1964). The classification of *R. solanacearum* into races and biovars is superficial and not give any phylogenetic information. Few years ago, based on sequence analysis of the internal transcribed spacer

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region, Fegan and Prior (2005) proposed a new classification scheme. *R. solanacearum* was subdivided into four phylotypes.

Today, although bacterial wilt disease is known to be widespread in Africa, the distribution, the characterization, and the genetic diversity of *R. solanacearum* strains in the continent in general and in Benin in particular, is scarcely documented. Strains were reported from Ethiopia (Lemessa and Zeller, 2007), Nigeria (Osuinde and Ikediugwu, 2002), Uganda (Osiru et al., 2001), Kenya (Ateka et al., 2001), Cameroon (Mahbou Somo Toukam et al., 2009) and Benin (Sikirou et al., 2009). They were also reported from Burundi, Egypt, Libya, Rwanda, South Africa and Tanzania, (Elphinstone, 2005; Fortnum and Kluepfel, 2005). African strains from Reunion, Island, Madagascar, Zimbabwe, and Angola have been characterized (Poussier et al., 1999).

In Benin the distribution and the bacterial wilt are poorly documented and the *R. solanacearum* strains have not been characterized.

Understand local pathogen diversity is capital to succeed breeding and integrated pest management program (Sanchez Perez et al., 2008).

The objectives of the present work were to (i) assess the incidence of tomato bacterial wilt in the field, (ii) study the distribution of *R. solanacearum* in Benin, and (iii) determine the pathogenic and biochemical characteristics of strains of *R. solanacearum* collected from different locations in Benin.

Materials and methods

Survey and incidence of tomato bacterial wilt

A comprehensive survey of bacterial wilt incidence was conducted throughout the 12 districts of Benin grouped in 8 agro-ecological zones (Fig. 1). Three plots of 50 m^2 (5 m × 10 m) were delimited in each of the two fields within three villages in three townships per district; the number of plants per plot varied between 50 and 200 according to farmers' cropping systems. Bacterial wilt incidence was assessed as the number of wilted plants out of the total number of plants per plot.

Origin and collection of strains

In each of the 72 fields surveyed, five wilted tomato plants were uprooted at random for bacterial isolation. The collected diseased samples were treated each day as follow after the survey. The stems were cut at the collar and the leaves were removed. Each stem was surface sterilized with 70% ethanol and washed with sterile distilled water. Five centimeters of the stem were cut from the lowest part, transversely cut into two parts then vertically divided into two or four sections. The stem pieces were soaked in a capped bottle containing 5 ml sterilized distilled water for 30 min to allow bacteria to diffuse into the water (Wullings et al., 1998), then removed with sterilized forceps. Fourfold serial dilutions of each sample were made in sterile water and aliquots of 100 μl were plated on triphenyltetrazolium chloride (TTC) medium (Kelman, 1954). Plates were incubated for 48 h at 30 °C. Presumptive *R. solanacearum* colonies were purified by streaking on a new TTC medium and stored in sterile distilled water in 1.5 ml pipette tubes (Sarstedt D. 51588 Number Drecht) at room temperature [Wullings et al., 1998; Kelman and Person, 1961) and in 20% diluted glycerin at −86 °C.

Strain identification

Strains were initially selected based on similarity in appearance to *R. solanacearum* of known strains on TTC medium. Presumptive

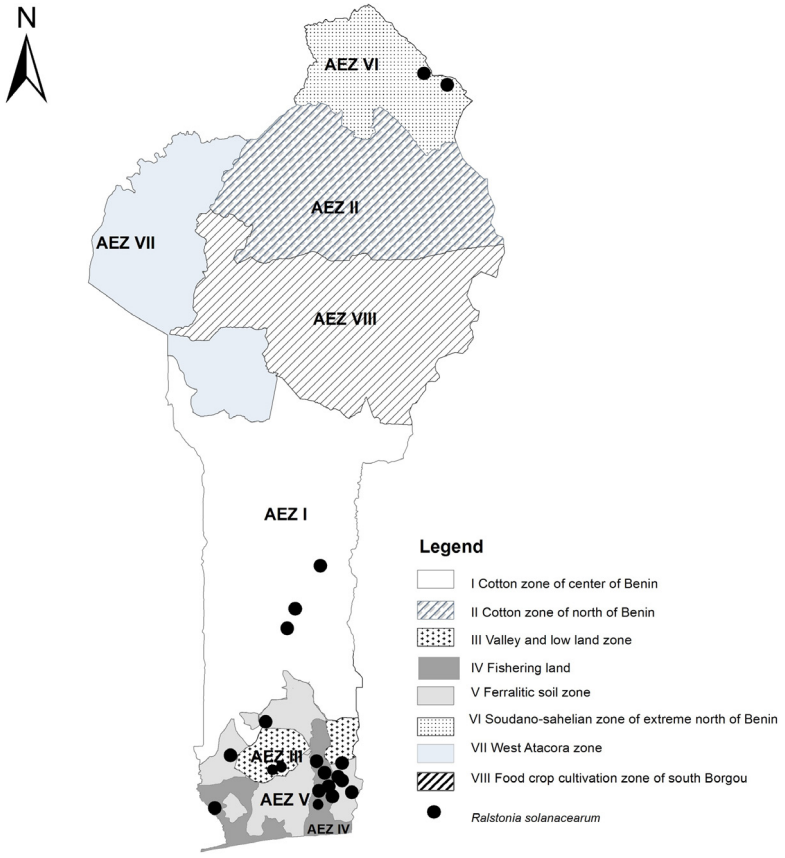

Fig. 1. Map of Benin showing the eight agro-ecological zones and the locations where *Ralstonia solanacearum* strains were collected.

R. solanacearum strains were purified by re-streaking one time on TTC medium, and then tested by ELISA (Pathoscreen Rs, Agdia Inc., Elkhart, IN USA) according to a modified method of Rajeshwari et al. (1998). The double antibody sandwich ELISA, based on monoclonal antibodies specific to *R. solanacearum*, was used according to the manufacturer's instructions. Bacterial suspensions ($\sim 10^8$ CFU/ml) were prepared from one-day-old cultures grown on Nutrient Broth Glucose medium. Wells in which color development was visibly darker than the negative control were recorded as positive for *R. solanacearum*.

Pathogenicity of strains

Seeds of the local tomato cultivar "Tohounvi" were obtained from the vegetable unit of Benin National Agricultural Research Institute (INRAB) and sown at the International Institute of Tropical Agriculture (IITA, Benin) in a plastic tray filled with field soil previously pasteurized for 1 h at 80 °C. Seedlings were watered every day as needed. Cultures of 76 *R. solanacearum* strains including a reference strain (reference strain ToUdk (race 1 biovar III; N. Thaveechai, Kasetsart University, Bangkok, Thailand) were grown on TTC medium for 2 days at 30 °C, then harvested from agar plates by flooding with sterile distilled water. Bacterial suspensions were adjusted to an optical density of 0.06 at 660 nm wavelength corresponding to $\sim 10^8$ colony-forming units per milliliter (CFU/ml).

Five tomato plants of 3-week-old per strain were transplanted into individual plastic pots (14 cm × 16 cm) containing sterilized field soil, and inoculated the same day by soil drenching with 30 ml of the test cultures as described by Diogo and Wydra (2007). Plants inoculated with sterile water served as control. The soil was watered before the transplantation and 24 h after inoculation.

Inoculated plants were incubated in a glasshouse at 21.6–30.3 °C and were closely monitored for wilt disease. Evaluations were carried out for 25 days. Four pathogenicity groups were determined: 1: wilt of plants within 7 days after inoculation (highly virulent), 2: wilt appeared between 7 and 15 days after inoculation (moderately virulent), 3: wilting occurred after 15 days (weakly virulent), 4: plants did not wilt (non-pathogenic). The test was repeated once for all strains except the strain 65c and N16.

Biovars of R. solanacearum strains from Benin

Carbohydrate oxidation

Biovar test was performed according to the modified mineral medium of (Ayers et al., 1919) (gl^{-1} $NH_4H_2PO_4$, 1; KCl, 0.2; $MgSO_4$ $7H_2O$, 0.2; Peptone, 1; Agar, 3; Bromothymol blue, 0.08; pH, 7.1 was used. The medium was heated to melt the agar and dispensed into Erlen Mayer and sterilized by autoclaving at 121 °C for 30 min and cooled to 60 °C. Ten milliliter of 10% aqueous solutions of the following carbohydrates (Dextrose, Mannitol, Sorbitol, Dulcitol, Trehalose, Lactose, Maltose, Cellobiose, Nitrate) were added to the medium (90 ml) to give a final concentration of 1%. After mixing, 3 ml of the molten medium was dispensed into sterilized test tubes and allowed to solidify. Twenty microliter of a test cultures were added to the surface of the medium in individual test tube (Ayers et al., 1919). The non-inoculated test tubes served as control. The inoculated and the non-inoculated test tubes were incubated at 31 °C. Tubes were examined at 3, 7, 14 and 28 days after inoculation. The changing of the green color to yellow indicates the positive result.

Statistical analysis

ANOVA were performed on disease incidence using the GLM procedure of SAS (SAS Institute Inc., Release 8.1, Carry, NC, USA). The Student-Newmann-Keuls test was used to compare mean values of incidence ($P \leq 0.05$). Values given in tables are means.

Results

Distribution of bacterial wilt in Benin

Bacterial wilt is widely distributed in Benin. *R. solanacearum* has been isolated from 8 districts including Plateau, Ouémé, Couffo, Atlantique, Zou, Alibori, Collines and Mono out of the 12 districts that the republic of Benin counts (Fig. 1). In the district of Plateau, tomato bacterial wilt has been found in the township of Adja-Ouèrè and Sakété. In the district of Ouémé, tomato bacterial wilt has been found in the townships of Adjohoun and Dangbo. In the district of Couffo, the disease has been only found in the township of Klouékanmey. In the district of Atlantique, the disease has been found in the township of Toffo.

In the district of Zou, it has been only identified in the township of Bohicon. In the district of Alibori, the disease has been found in the township of Karimama and Malanville.

In the district of Collines, bacterial wilt has been found in the township of Savè and Dassa. In the district of Mono, the disease has been found in the township of Athiémé (Table 2). The corresponding infected villages of each township state in Table 1.

Based on the agro-ecological zones, *R. solanacearum* was isolated from 5 out of the 8 agro-ecological zones which belong to the major tomato cropping area. Bacterial wilt was more severe in AEZ V (ferralitic soil of Sakété and Adjaouèrè), AEZ IV (valley of Adjohoun, Dangbo, Hêtin, Athiémé and the low land of Klouékanmey) and AEZ I (highland of Savè). No *R. solanacearum* strains were obtained from AEZ II, AEZ VII and AEZ VIII (Fig. 1).

Incidence of bacterial wilt in Benin

A total of 313 wilted plants were collected from 156 fields in 12 districts. Of these, 75 were infected by *R. solanacearum*; the other 238 plants were infected by *Sclerotium rolfsii* (149), *Fusarium oxysporum* (23) and *Fusarium solani* (5). Wilting of the remaining 61 plants was attributed to drought and inadequate application of mineral fertilizer. The incidence of bacterial wilt was significantly different among villages ($P < 0.0001$), townships ($P < 0.0001$) and districts ($P < 0.0001$). Incidence was high in the districts of Couffo AEZ V (71.6%), Mono AEZ IV (48.3%), Ouémé AEZ IV (33.8%), Plateau AEZ V (30%) and Collines AEZ I (18%) and lower in that of Alibori AEZ VI (2%), Zou AEZ III (7%) and Atlantique AEZ III (14%) (Table 1).

Among the townships, bacterial wilt incidence was high in the township of Klouékanmey AEZ V (71.6%), Athiémé AEZ IV (48%) and Dangbo AEZ IV (40.7%) and low in that of Malanville AEZ VI (0.8%), Karimama AEZ VI (2.7%) and Bohicon AEZ V (7.4%). The incidence in the villages ranged from 0.5 to 71.6 % (Table 1).

Identification of isolated strains

Pathological characterization of strains

All the 137 putative *R. solanaceraum* strains collected from the field were similar in colony type on TTC medium to known *R. solanacearum* strains (ToUdk race 1 biovar III; N. Thaveechai, Kasetsart University, Bangkok, Thailand). However, only 76 strains including the reference strain caused typical symptoms on tomato. The remaining 62 strains were non-pathogenic. With the pathogenicity test, wilting appeared between 4 to 17 days after inoculation. Forty-one (41) strains caused plant wilt within 7 days after inoculation and were classified as highly virulent. Twenty-seven (27) strains caused the wilt of plants between 7 and 15 days

Table 1
Field incidence of tomato bacterial wilt caused by *Ralstonia solanacearum*.

AEZ[a]	Bacterial wilt incidence by district (%)		Bacterial wilt incidence by township (%)		Bacterial wilt incidence by village (%)	
V	Plateau	[b]30.0bc	Adja-Ouèrè	28.7bc	Tatonnonkon	55.0abc
					Ikpilè***	2.5e
			Sakété	31.3bc	Itadjèbou-Igboabikoun***	41.7abcde
					Itadjèbou-Igboassan	21.0cde
IV	Ouémé	33.8bc	Adjohoun	26.9bc	Dannou***	18.6cde
					Gogbo***	28.2bcde
					Gangban***	34.0abcde
			Dangbo	40.7bc	Agonguè***	68.3ab
					Hêtin***	53.3abcd
					Gbéko*	0.5e
V	Couffo	71.6a	Klouékanmey	71.6a	Toïmey***	71.6a
IV	Mono	48.3b	Athiémé	48.3ab	Athiémé-Center***	48.3abcde
III	Atlantique	14.1c	Toffo	14.1bc	Akpé***	26.7bcde
					Akpé-liho	7.9de
					Ouègbo***	21.8cde
V	Zou	7.4c	Bohicon	7.4c	Houèssouho*	7.4de
I	Collines	18.0c	Savè	34.1bc	Gobé-Atchakpa	34.1abcde
			Dassa	12.6bc	Gomey***	23.8cde
					Odo-Otchère***	11.7cde
					Godogossou	2.2e
VI	Alibori	1.7c	Karimama	2.7c	Kargui*	2.7e
			Malanville	0.8c	Noureni*	0.8e
	P < 0.0001		P < 0.0001		P < 0.0001	

[a] AEZ = agro-ecological zone of Benin.
[b] Means within column with the same letters are not significantly different according to Student-Newmann-Keuls test with $P = 0.05$.
* Village with low virulent strains.
*** Village with highly virulent strains.

Table 2
Pathogenicity of strains of *R. solanacearum* from Benin on tomato and their classification into pathogenic group, biovar and race.

Strains	Original host	Biovar	Pathogenicity group	Race
67S, 68S, 69S,	Tomato	I	1	1
70S, 71S	Tomato	I	2	1
46S, 47S, 49S, 50S, 51S, 52S, 55S, 56S, 57S, 63S, 64S 65S, 100CS, 44C, 46C, 47C	Tomato	III	1	1
77S, 78S, 79S, 80S, 81S, 10V, 56V, 63V, 66V, 15V, 25V, 32V, 99AS, 99BS, 100AS, 100BS, 114S, 45C, 49C, 43C, 21C, N17, N18, 86C, 77C, 104C	Tomato	III	2	1
2V, 3V, 4V, 6V, 62V, 67V, 26V, 115S, 116S, 117S, 15C, 118S, 25C, 31C, 72C, 75C,	Tomato	III	1	1
71V, 12V, 14V	Pepper	III	1	1
72V	Pepper	III	2	1
73V, 65V, 39V, 44V, 19C, 65C, N33	Tomato	III	3	1
N16	Eggplant	III	3	1
ToUdk (reference strain)[a]	Tomato	III	2	1

1 = highly virulent: strains causing the wilt of plants within 7 days after inoculation.
2 = moderately virulent: strains causing the wilt of plants between 7 and 15 days after inoculation; 3 = weakly virulent: strains causing the wilt of plants after 15 days of inoculation.
Strains 65c and N16 were tested once.
[a] Strain from Thailand.

after inoculation and were classified as moderately virulent and eight (8) strains caused the wilt of plants after 15 days of inoculation and were classified as weakly virulent. The strains 71v, 72v, 12v and 14v isolated from pepper were highly virulent on tomato while the strain N16 isolated from *Solanum melongena* was weakly virulent on tomato (Table 2). Highly virulent strains were isolated from all 5 AEZ where bacterial wilt of tomato was present except the northern part of AEZ VI where all isolated strains were weakly virulent (strains N16, N3) (Table 2).

ELISA

All the 76 virulent *R. solanacearum* strains were positive by ELISA test based on visual observation of color development. Seven non-pathogenic strains were tested negative for *R. solanacearum* by ELISA.

Biovars and race of R. solanacearum strains from Benin

Carbohydrate oxidation

All 76 *R. solanacearum* strains that were pathogenic on tomato and tested positive by ELISA utilized dextrose, cellobiose and trehalose. However, marked and significant differences were observed in the ability of the strains to utilize manitol, sorbitol and dulcitol. Differences were also observed in their ability to oxidize lactose and maltose. None of the strains produced gas and nitrite from nitrate. No reaction was produced in inoculated media without a carbohydrate source. Seventy one of the 76 strains were classified as biovar III and five as biovar I according to the classifi-

cation scheme of Hayward, 1964. The biovar III strains were distributed in all the 5 AEZ and the biovar I was found in one village of AEZ V. Both biovar I and III belong to race 1.

Discussion

In Benin bacterial wilt caused by *R. solanacearum* has been found from the south to the north. It was more disseminated in the south than the center and the north of Benin where it was only found in an area where potato is often cultivated. As in the Amazonas states (Coelho Netto et al., 2003), *R. solanacearum* has been isolated in Benin from the valley a periodically flooded fields (Dannou, Gogbo, Gangban, Agonguè, Hêtin, Gbéko, Athiémé, Toïmey, Kargui and Nouréni), from non-flooded fields (Tatonnonkon, Ikpilè, Itadjèbou, Igbo-Assan, Akpé, Akpé-liho, Ouègbo-Center, Houessouho and Godogossou) and from the low land (Gobé-Atchakpa, Gomey and Odo-otchèrè). The highest incidence of the disease was recorded in the southern part of Benin particularly in the periodically flooded area where more than 70% of wilted plants were recorded. This result explain that the soil of the periodically flooded area is high concentrated in inoculum which came from streaming water.

This high incidence of *R. solanacearum* impairs the production of tomato especially in the valley, the most periodically flooded area in Benin. One possible justification of this is the reduction of tomato cultivated area from 1502 ha to 1232 ha and from 370 ha to 132 ha in the township of Dangbo and Athiémé respectively from 1999 to 2004 (MAEP, 2004). The same data trend was observed for tomato yield which decreased from 5012 kg ha^{-1} to 406 kg ha^{-1} and from 6000 kg ha^{-1} to 400 kg ha^{-1} respectively in the township of Adjohoun and Dangbo from 1999 to 2004 (MAEP, 2004).

In the central part of Benin, the incidence of tomato bacterial wilt was about 34% in the highest infected fields. In the north, the incidence of the disease was less than 3%. This result demonstrates that the bacterial wilt was scarce in the north which is the biggest tomato producing area of Benin during the dry season. The strains N16, N17 and N18 were isolated from locations where potato was often cultivated in the north. This assumes that the occurrence of *R. solanacearum* in the north of Benin may be due to the introduction of potato planting materials. This result indicates that the north part of Benin is almost free of *R. solanacearum* except some part in the villages of Kargui and Noureni where potato is produced. Therefore, the north is an appropriate area for the production of tomato or other solanaceous crops in Benin. This involves the strict regulation on the introduction of the diseased potato planting material.

Based on the agro-ecological zones, *R. solanacearum* was isolated from 5 out of the 8 agro-ecological zones which belong to the major tomato cropping area. The 5 AEZ includes in largest part of the valley, and the ferralitic soil and in few part the high land. Bacterial wilt was more distributed and more severe in the low lands and the valley of rivers which are flooded areas than in the highland and ferralitic soil. This result corroborated that of Prior and Fegan (2005) and (Coelho Netto et al., 2003) who isolated *R. solanacearum* strains from high and low land of Cameroon and Amazonas respectively.

In the present study, the identification of *R. solanacearum* strains of Benin was performed using different methods including morphological characteristics of the strains on TTC medium, serological immunoscript tests, ELISA test and pathogenicity test. It was found that, the use of TTC medium alone as identification method of *R. solanacearum* is not enough, although it is less costly and simple. The study showed that some saprophytic bacteria with closely related colony appearance to *R. solanacearum* could limit the efficiency of TTC medium. However pathogenicity and ELISA tests could be used as identification methods as recommended by EU (1998).

All collected *R. solanacearum* strains and the reference one on TTC medium resembled those strains from other regions of the world Kelman, 1954; He et al., 1983; Williamson et al., 2002; Lemessa, 2006. Some strains produced fluidal colonies and some non-fluidal colonies but both with a red center on TTC medium (Dannon, 2003).

The biochemical test demonstrated that the Beninese *R. solanacearum* strains belong to biovar I and biovar III both belong to race 1 (Hayward, 1994). As reported by many studies, biovar I doesn't utilize Cellobiose (Lemessa, 2006; Schaad et al., 2001). In our study, strains that are close to biovar I utilized Cellobiose. Strains of biovar I were isolated from tomato and strains of biovar III were isolated not only from tomato but also from pepper (*Capsicum frutescens*) and *Solanum melongena*. Biovar or race of *R. solanacearum* strains from Benin was unknown. With this study biovar I and biovar III strains of *R. solanacearum* are the first report from Benin.

Most of the isolated strains were highly pathogenic to tomato depending on whether isolated from tomato or pepper. This result confirms many other results which had classified *R. solanacearum* strains isolated from pepper and tomato in the same biovar and race (Hayward, 1994). In contrary, the strain isolated from *Solanum melongena* was less pathogenic to tomato. This could be explained by the host specificity reaction. Most of the isolated strains from the north were collected from potato and tomato growing fields and were less pathogenic to tomato. They might be highly pathogenic to potato. This indicates the poor incidence of bacterial wilt of tomato in the north of Benin since strains are not virulent.

The biovar of the tested strains did not depend on their pathogenicity. High, medium or low pathogenic strains belong to biovar III and biovar I. All pathogenic strains whatever their group had given positive result with ELISA test. This confirms the strains to be *R. solanacearum.*

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