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Virulence and characterization of isolates of potato bacterial wilt caused by *Ralstonia solanacearum* (Smith) in Rwanda

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Bacterial wilt (*Ralstonia solanacearum*) is one of the major potato diseases in Rwanda. An *in vitro* study was carried out to identify and characterize the pathogen isolated from three potato cultivars (Kinigi, Kirundo and Gikungu) in Rwanda. This was achieved by cultural and morphological tests on triphenyl tetrazolium chloride (TTC) and casamino peptone glucose (CPG) agar as well as biochemical tests through Gram staining and biovar test. An *in vivo* experiment was also performed to assess the pathogenicity of those isolates on potatoes. All isolates showed typical morphological traits of virulent *R. solanacearum* on TTC and CPG media. The test isolates were Gram-negative bacteria. Biovar test confirmed that all the isolates belonged to race 1 biovar 3 of the pathogen. Furthermore, the highest disease severity (DS=100%) and disease incidence (DI=100%) were observed in Gikungu isolate followed by Kinigi (DS=97.33% and DI=98.25) and Kirundo (DS=94.67% and DI=92.61%). From this study, all three isolates were typical *R. solanaceraum* belonging to race 1 biovar 3 and were all pathogenic to potato plants. Gikungu and Kinigi isolates were highly virulent than Kirundo isolate. Therefore, Gikungu or Kinigi isolates can be used for further studies in plant protection in management of the disease.

Key words: Biovar test, Gram-negative, Gram-positive, pathogenicity test.

INTRODUCTION

Potato is the world's most widely grown tuber crop (Strange and Scott, 2005; Were et al., 2013) and the fourth major food crop of the world after rice, wheat and maize (Wagura et al., 2011; Guchi, 2015). In Rwanda, potato is among the priority crops, one of the most important food and cash crops for small scale farmers especially in the Northern regions (REMA, 2011; RAB, 2012; Muhinyuza et al., 2014; Uwamahoro et al., 2018). Annual consumption of potato is around 125 kg per person per year and it is the country's second most important source of energy after cassava (REMA, 2011; Muhinyuza et al., 2014). Although Rwanda is the third

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most important potato producer in sub-Saharan African after Malawi and South Africa (FAO, 2014), average yield is estimated at 9 t ha^{-1} which is below the potential yield of 40 to 60 t ha^{-1} (Masengesho et al., 2012; Muhinyuza et al., 2014).

Pests and diseases are among the major limiting factors in potato production worldwide which decrease the production of the crop by 36% (Muhinyuza et al., 2007; Wagura et al., 2011; Yuliar et al., 2015). In Rwanda, brown rot or bacterial wilt caused by *Ralstonia solanacearum* (Smith) (Yabuuchi et al., 1995) is the second most destructive disease in Rwanda after potato late blight caused by *Phytophthora infestans (*Mont. De Bary) (Muhinyuza et al., 2007; REMA, 2011; Uwamahoro et al., 2018). Although late blight is ranked as a priority disease, its management can somehow be achieved by applying fungicides like Dithane M45 or Ridomil in combination with the use of resistant varieties (REMA, 2011; Uwamahoro et al., 2018). However, bacterial wilt should be considered as more problematic than late blight because it cannot be controlled by chemicals or agronomic practices like other main potato diseases (Wagura et al., 2011; Masengesho et al., 2012; RAB, 2012; Huet, 2014; Uwamahoro et al., 2018). This is due to the fact that there is no known chemical that can be used to control it once potato plant is infected because of its lethality, persistence, wide host range and broad geographic distribution (Strange and Scott, 2005; Guchi, 2015; Huet, 2014; Yuliar et al., 2015).

In addition, the application of cultural practices such as crop rotation, use of non-infected seeds, planting in non-infected soils and growing resistant cultivars have various limitations (Huet, 2014; Uwamahoro et al., 2018). For example, crop rotation is not effective because the pathogen can survive in the soil for long periods even in the absence of host plants and it also has a broad host range. Moreover, the disease has been observed even in first planting in newly cleared land. In addition, small farm size is also another challenge for crop rotation program. Furthermore, planting materials are exposed to bacteria attack because potato is mainly propagated vegetatively, and this method favors pathogen spread from mother tubers to the next crop generation. The use of resistant varieties is also limited because they are scarce, some of them are not appreciated by farmers, and they can harbor the latent infection in tubers (Priou et al., 2001; REMA, 2011; Wagura et al., 2011; Huet, 2014; Yuliar et al., 2015; Uwamahoro et al., 2018).

R. solanacearum is a non-spore forming, non-fluorescent species of the genus *Ralstonia*. It is a Gram-negative bacterium, strictly aerobic with round-shaped cells, 0.5 to 1.5 µm in length, with a single, polar flagellum (Hayward, 1991; Priou et al., 2001; Stevenson et al., 2001). The pathogen enters plant through wounds or stomata. Once inside the plant, it moves towards the vascular bundles, and finally colonizes the xylem. The presence of the bacteria inside the xylem is coupled with the production of exopolysaccharides which block the vascular vessels inducing a water shortage throughout the plant (CIP, 1996; Muthoni et al., 2012; Rahman et al., 2012; Guchi, 2015). External disease symptoms of *R. solanacearum* in potato are mainly wilting of the plant and droplets of bacterial ooze from the eyes of the potato tubers. The internal symptoms are brownish discoloration of the vascular ring inside the tuber and grayish white droplets of bacterial cream which come out of them (Priou et al., 2001; Pradhanang et al., 2003; Strange and Scott, 2005; Muthoni et al., 2012; IPDN, 2014; Guchi, 2015).

R. solanacearum is usually closely associated with its living host plants mainly solanaceous crops and temporarily in infected host-plant debris or host weeds. This plant pathogenic bacterium survives for relatively a period of five to eight years in soil or other environment where there are host plants or weeds like *Datura* and *Portulaca* (Hammes, 2013). In the soil, the pathogen may remain there almost indefinitely because it can survive saprophytically and/or it can parasitize a number of very common weeds (REMA, 2011; Uwamahoro et al., 2018). Host debris, latent infected tubers and deeper soil layers were most important factors for its survival (Muthoni et al., 2012; Hammes, 2013). Various environmental factors such as temperature, moisture and rainfall, soil type, inoculum potential, and other soil biological factors such as nematode populations have been reported to correlate with development rate, survival and incidence of pathogen (Hayward, 1991; Uwamahoro et al., 2018).

The isolates of this bacterium can be classified into five different races based on the host range and five biovars on the basis of their ability to utilize the disaccharides (cellobiose, lactose, and maltose) and to oxidize the hexose alcohols (dulcitol, mannitol and sorbitol) (Muthoni et al., 2012; IPDN, 2014). Race 1 of *R. solanacearum* affects a wide range of plant species in the Solanaceae family including potato, tomato and eggplant. It has been observed that *R. solanacearum* race 2 affects some plants of the Musaceae family such as banana and plantains. Race 3 affects mainly potato and tomato and to a less extent to other solanaceous species. Race 4 affects particularly ginger whereas race 5 affects mulberry trees (CIP, 1996; OEPP/EPPO, 2004; Sikirou et al., 2017). In addition, there is also a relationship between biovars and races of *R. solanacearum* and their location. This means that each race contains specific biovars and the races can also adapt to different regions due to the specific requirements in climatic conditions for their survival (Muthoni et al., 2012; Sikirou et al., 2017).

Although Rwanda is one of African countries where bacterial wilt is threatening potato production, the pathogen is poorly studied and its management is getting more difficult due to the challenges mentioned earlier. Identification of the biovars and characterization of the

Figure 1. Symptoms of potato bacterial wilt (*Rasltonia solanacearum*) on potato plants and tubers collected from Kinigi site. Stem/Leaf wilting of Kinigi, Gikungu, and Kirundo cultivars (A, B, and C, respectively). Bacterial ooze and soil clumps on eyes of Kinigi, Gikungu, and Kirundo cultivars (D, E, and F, respectively). Brown ring and ooze from tubers of Kinigi, Gikungu, and Kirundo cultivars (G, H, and I, respectively).

isolates is important for example in management of the disease because there is a correlation between the biovar type, race and ultimately the host range of *R. solanacearum* as well as the climatic conditions it may adapt to (Fock et al., 2001; Strange and Scott, 2005; Popoola et al., 2015). In addition, deep understanding on how different isolates may be virulent on plant hosts can also provide the main key in application of adequate control measures of the pathogen such as creation of potato resistant cultivars. The objectives of this study were, therefore, to identify the biovars of different bacterial isolates and to evaluate their pathogenicity and virulence on potato plants.

MATERIALS AND METHODS

Origin of bacterial isolates

Three isolates of *R. solanacearum* were collected from three infected and most susceptible potato cultivars grown in Rwanda namely Kinigi, Kirundo, and Gikungu with typical bacterial wilt symptoms as shown in Figure 1. The isolates were collected from farmers' fields at Kinigi in November 2017. Kinigi is located in the highland of volcanic soils at an altitude of 2,300 m above sea level (m.a.s.l.), with low temperature (average of 20°C) and high rainfall (1,400 mm to 1,800 mm and well distributed throughout the year) (Birasa et al., 1990; Lepoint and Maraite, 2002; RAB, 2012; MINIRENA, 2013).

Isolation, identification and storage of isolates

All activities for isolation, culturing and identification of bacterial isolates were done in the Plant Pathology Laboratory of Rwanda Agriculture Board (RAB)-Northern zone at Musanze. A vascular flow test and observation of natural slime drop formation after cutting tubers or plants are unique to *R. solanacearum* (Chaudhry and Rashid, 2011; Priou et al., 2001; IPDN, 2014). In this study, diseased tubers from Kinigi, Kirundo and Gikungu potato cultivars collected from grower's fields were used. Tubers were first washed in tap water, then surface sterilized by soaking them in 70% ethanol solution for 5 min and well rinsed three times in distilled water and left to dry. Then, tubers were cut into circular pieces which were suspended for 10 min in sterile distilled water in a glass container to

detect the presence or absence of exudation from the tubers.

Cultural, morphological, and physiological characteristics of single colonies were determined on Petri plates containing specific culture medium. Kelman's Triphenyl Tetrazolium Chloride (TTC) and Casamino peptone glucose (CPG) agar were used as selective media for isolation, identification and biochemical characterization of *R. solanacearum* (Kelman, 1954; IPDN, 2014). Culture of bacteria on both TTC and CPG was achieved by fivefold serial dilution in order to estimate the number of bacteria cells or colony forming units (CFU) in 1 mL of original inocula from three bacterial isolates. Initial bacterial inocula were obtained by collecting suspension of bacterial streaming in sterile distilled water through a vascular flow technique as described earlier. Serial dilution was done from 10^{-1} up to 10^{-5} dilution factor in five tubes each filled of sterile nutrient broth containing peptone, sodium chloride, meat and yeast extracts as described by Marangoni et al. (2001). Each suspension of serially diluted cells were plated on TTC and CPG agar media and incubated at 28°C for 48 h, a period after which bacterial colonies were counted and calculation of CFU mL^{-1} was performed by the following formula (IPDN, 2014):

CFU mL^{-1} = (number of colonies × dilution factor) / volume of culture plate

Counting of the colonies was possible at dilution factor of 1:100,000 (plate labeled as 10^{-5}) on both TTC and CPG media. From the counted colonies, the CFU mL^{-1} in the original bacterial suspension ranged from 4.3×10^7 for Kirundo to 4.8×10^7 for both Gikungu and Kinigi bacterial isolates, respectively on TTC agar and from 4.8×10^7 for Kirundo and Kinigi to 4.9×10^7 for Gikungu isolate on CPG agar.

Purification of bacterial colonies was achieved through culturing two times single colony of virulent *R. solanacearum* isolated from TTC and CPG media on new TTC or CPG growth media. The colonies or cell mass were transferred into a sterile glycerol stock (80% of glycerol mixed with 20% of nutrient sucrose broth) in which a loop full of two days old colonies from TTC or CPG were transferred and kept at -20°C for subsequent uses (IPDN, 2014). A further diagnosis was performed to distinguish this Gram-negative bacterium from Gram-positive bacteria and this was achieved by simple Gram staining as described by Chaudhry and Rashid (2011).

Pathogenicity test

Pathogenicity test for the three bacterial isolates recovered from different potato cultivars (Kinigi, Kirundo and Gikungu) was carried out by soil inoculation. Healthy potato seeds of Kirundo, which is the most susceptible potato cultivar to *R. solanacearum,* were obtained from RAB, Kinigi station. After washing and surface sterilizing the tubers, the later were planted in plastic pots filled with pasteurized mixture of soil, organic matter and sand (2:1:1) and grown under greenhouse conditions (16.7 to 37.4°C temperature and 31 to 75% relative humidity) at RAB, Musanze. Bacterial inoculation was done when seedlings were 30 days old. Soil around plant roots was removed, and then half of the roots of each potato plant were slightly cut. Then 10 ml of bacterial suspension at concentration of 4.8×10^7 CFU mL^{-1} were inoculated around the roots of each pot. Seedlings sprayed with sterile water served as control.

This test was performed using a randomized complete block design (RCBD) in which the pathogenicity of three bacterial isolates was defined as treatments in one potato cultivar. Each treatment was replicated three times (blocks). In each block, five potato seedlings were inoculated with each bacterial isolate or sterile water that served as the control. This means that 20 potato plants per block were used and a total of 60 plants for all the greenhouse experiment were used. Disease incidence (DI %) and disease severity (DS %) in potato plants were evaluated starting from the appearance of symptoms (five days after inoculation) until all the plants inoculated with the most virulent bacterial isolate died. The disease severity (DS %) in plants was evaluated using the scale of Kempe and Sequeira (1983), where 0 = no symptoms; 1 = 1 to 25% leaves wilted; 2 = 26 to 50% leaves wilted; 3 = 51 to 75% leaves wilted; 4 = more than 75% but less than 100% of leaves wilted; 5 = all leaves wilted and plant dead. The severity was calculated using the following formula (Kempe and Sequeira, 1983):

$$DS\ \% = [\Sigma (ni \times vi) \div (V \times N)] \times 100$$

where DS = Disease severity; *ni* = number of plants with the respective disease rating; *vi* = disease rating; *V* = the highest disease rating; and *N* = the number of plants observed.

Disease incidence (DI) was evaluated by the following formula (Kempe and Sequeira, 1983):

$$DI\ \% = \frac{n}{N} \times 100$$

where DI = Disease incidence; n = number of infected leaves per plant; and N = total number of leaves per plant.

From the diseased potato plants, the bacteria were re-isolated and cultured on TZC media and CPG to confirm the presence or absence of the typical colonies of *R. solanacearum* to proof Koch's postulates as well as for further uses.

The analysis of variance (ANOVA) was carried out using SAS software, to determine the difference in wilt incidence and wilt severity due to the three isolates of the pathogen. The treatments means were separated using Tukey's honestly significant difference test at $P \leq 0.05$.

Biovar identification

Biovar determination of the three bacterial isolates was done on both the isolated bacteria strains from Kinigi site and the re-isolated ones from diseased plants during pathogenicity test which was performed under greenhouse conditions. The biovars were determined based on the ability of isolates to oxidize hexose alcohols namely dulcitol, mannitol and sorbitol or to utilize disaccharides like cellobiose, maltose, and lactose. Basal medium as described by Sikirou et al. (2017) was composed of 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1 g of peptone, 0.03 g of bromothymol blue, and 3 g of agar per 1 L of distilled water. The medium was sterilized by autoclaving at 121°C for 15 min. After sterilization, 10% of sugar or alcohol solutions preliminary sterilized by boiling them in water bath for 20 min for three successive days were amended to the basal medium. Then, this mixture was dispensed on 96 well plates by pouring 200 µl of medium in each well. Sterile distilled water served as control. Thereafter, 20 µl of bacterial suspension at concentration of 4.8×10^7 CFU mL^{-1} were added to each well. The plates were incubated at 28°C for seven days.

Utilization of sugars and oxidation of alcohols were shown by a positive (+) reaction which leads to changing in color from green (initial color of medium) to yellow. Otherwise it remains green (-) because the bacterial strains do not utilize the test sugars or oxidize the alcohols (Muthoni et al., 2012; Sikirou et al., 2017). During this experiment, the results of color change of the medium were visually observed from four to seven days. The experimental was set up in completely randomized design (CRD) with three treatments that

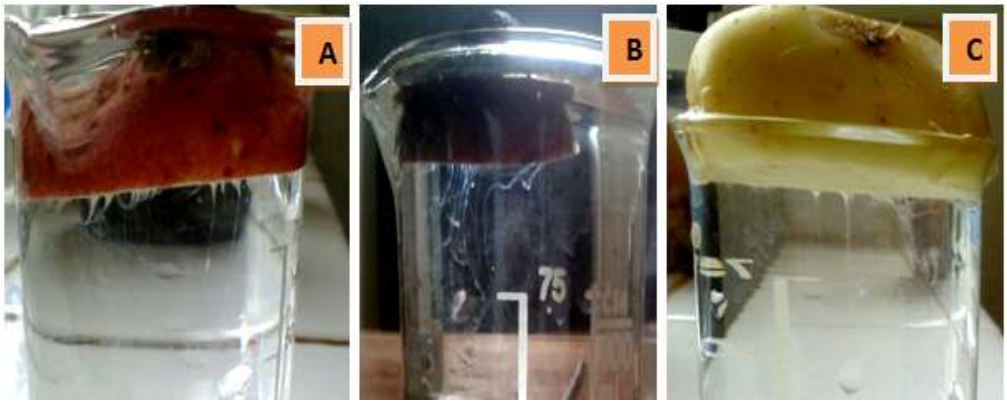

Figure 2. Identification of isolated *R. solanacearum* by vascular flow test: Bacterial streaming characterized by smoke-like milky exudates from infected tubers of Kinigi (A), Gikungu (B), and Kirundo (C) cultivars.

corresponded to each bacterial isolate that was replicated four times.

RESULTS AND DISCUSSION

Bacterial streaming test

In this study, infected potato tubers from Kinigi, Kirundo and Gikungu cultivars were used to identify and confirm the presence of *R. solanacearum* in the samples. A vascular flow test was performed to confirm whether the isolated pathogen from infected potato plant materials was *R. solanacearum*. Other pathogens can also cause wilting symptoms on potato plants such as *Fusarium solani* (Mart. Sacc.), *Verticillium alboatrum* (Reinke and Berth), *Erwinia chrysanthemi* (Burkholder) and *Clavibacter michiganensis* subsp. *Sepedonicus* (Spieckermann and Kotthoff) (Priou et al., 2001; Nadia et al., 2013; El-Habbaa et al., 2016). Through this test, the presence of bacterial wilt in tubers was characterized by smoke-like milky stream that streamed downward from all the cut tubers (Figure 2). Typically, this streaming differentiates *R. solanacearum* from other bacteria which may lead to similar symptoms in potatoes in which these threads are not formed (Priou et al., 2001; Nadia et al., 2013; El-Habbaa et al., 2016). Since the results confirmed the presence of *R. solanacearum*, each isolate was named according to the cultivar that they were isolated from Kinigi, Kirundo and Gikungu respectively.

Morphological features of colonies on growth media

Shape, size and color of colonies on TTC and CPG media are other characteristics which are used to identify the pathogen and to distinguish the virulent and non-vilurent colonies of *R. solanacearum* (Kelman, 1954; Nadia et al., 2013; Sikirou et al., 2017). The colonies which developed on TTC growth medium were fluidal, big, irregularly shaped, and white with pink or red colored center whereas on CPG, they were also big with irregular shape and white color (Figure 3). These features of bacteria on both TTC and CPG agar media confirmed that all the test isolates had typical morphological and cultural characteristics of *R. solanacearum* and were able to infect potato plants and to lead to plant wilting. The same bacterial traits of *R. solanacearum* among other bacteria on culture media were confirmed by Priou et al. (2001), Nadia et al. (2013), IPDN (2014), and Sikirou et al. (2017). These traits are different from non-virulent colonies which are usually smaller, dry, and uniformly dark-red on TTC agar medium and smaller, regularly round and dry on CPG agar (Narasimha and Srinivas, 2012; Muthoni et al., 2012; Nadia et al., 2013; IPDN, 2014; El-Habbaa et al., 2016).

Morphological characteristics of bacterial cells through gram staining

Gram staining was performed to confirm the gram type of *Ralstonia* isolated from Kinigi, Kirundo and Gikungu potato cultivars. From the results of this test, the microscopic observation showed that bacterial cells stained reddish (Figure 4) and this confirmed that the isolated pathogen was a Gram-negative bacterium and distinguished *R. solanacearum* from Gram-positive bacteria which are usually stained purple by this test (Hayward, 1991; Chaudhry and Rashid (2011); Nadia et al., 2013; Popoola et al., 2015). In addition, isolated bacterial cells were rod shaped (Figure 4) and this observation also supported the fact that the isolated pathogen was *R. solanacearum* since its round shape

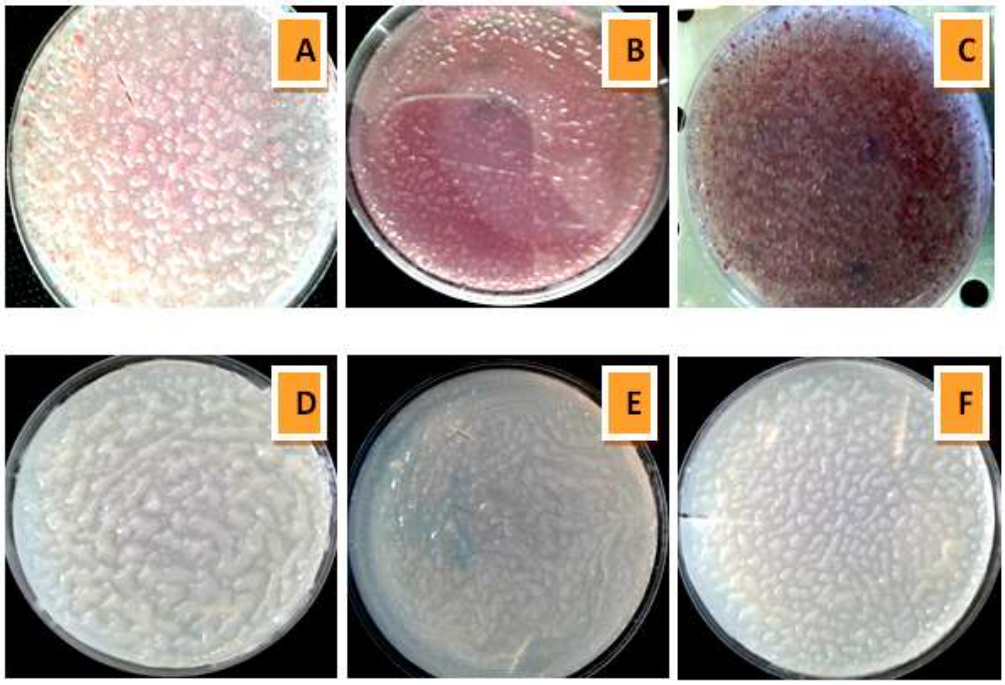

Figure 3. Morphological features of colonies of *R. solanacearum* on TTC and CPG culture media. (A, B, C) Kinigi, Gikungu, and Kirundo isolates respectively on TTC agar at serial dilution 10^{-3}: colonies are mucoid, big with irregular shape, and white with pink colored center. (D, E, F) Kinigi, Gikungu, and Kirundo isolates respectively on CPG agar at serial dilution 10^{-3}: fluidal, big and irregular white shaped colonies.

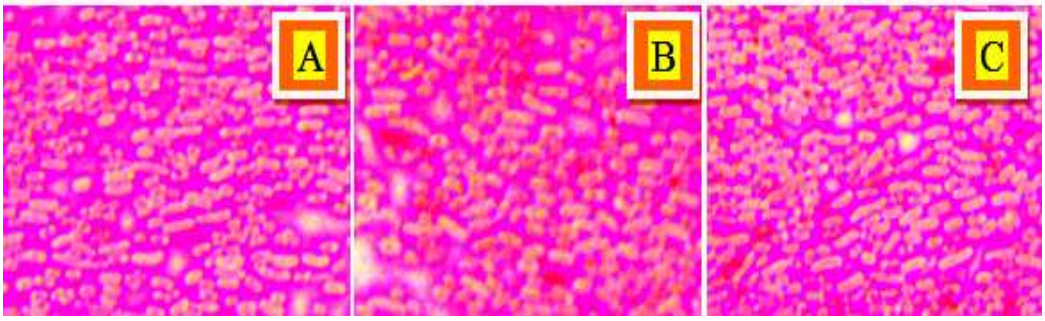

Figure 4. Gram staining pictures from microscopic observation of Kinigi, Gikungu, and Kirundo isolates (A, B, and C, respectively).

was confirmed by Hayward (1991), Stevenson et al. (2001), and Popoola et al. (2015).

Pathogenicity and virulence of the bacterial isolates

Pathogenicity of Kinigi, Kirundo and Gikungu bacterial isolates was tested based on wilting rate they caused to Kirundo potato seedlings under greenhouse conditions. From the wilting rate, disease incidence (DI) and disease severity (DS) were evaluated and calculated from 5 to 20 days after inoculation (5DAI - 20DAI) with an interval of 5 days. The results showed that at 5DAI (Figure 5, 1st row), inoculated plants started to wilt in potatoes treated with Kinigi, Gikungu and Kirundo isolates whereas the control plants did not show any symptom of bacterial wilt. Wilting rate of potato plants increased over time in seedlings inoculated with all test bacterial isolates especially from 10 DAI (Figure 5, 2nd row). Wilting was higher especially in potatoes inoculated with Kinigi and

Figure 5. Wilting of potato seedlings at 5, 10, 15, and 20 days after inoculation (1st, 2nd, 3rd, and 4th row, respectively) with *R. solanacearum* isolates.

Gikungu isolates than in plants inoculated with Kirundo isolate at 10, 15 and 20DAI (Figure 5, 2nd, 3rd, and 4th row respectively). At 20DAI all plants (100%) inoculated with Gikungu isolate died and almost all plants (98.25%) inoculated with Kinigi also died. During the whole experimental period, there was no wilting in plants treated with water which served as the control.

Potatoes treated with sterile water (control) did not show any symptoms of bacterial wilt. Wilting occurred in potatoes inoculated with Kinigi, Gikungu, and Kirundo bacterial isolates, respectively. Wilting of potatoes inoculated with bacterial isolates increased over time either within the isolate or between isolates. At 20DAI all plants inoculated with Gikungu isolate died.

In addition, based on the rates of the potato plant wilting, it was observed that DI as well as DS caused by the three bacterial isolates increased over time. Thus, from 5 to 10 DAI potatoes inoculated with Kinigi, Kirundo and Gikungu bacterial isolates resulted to DI and DS which increased over time but not significantly different between them at $P \leq 0.05$. However, from 15 to 20DAI, there was a significant difference at $P \leq 0.05$ in disease incidence and severity caused by the three bacterial isolates. From 15 up to 20DAI, Kinigi and Gikungu isolates caused higher disease incidence and severity in comparison with Kirundo isolate but with no significant difference between them. Furthermore, at 20DAI inoculation of potatoes with Gikungu isolate caused significant difference level of disease incidence and severity in comparison with Kirundo while Kinigi did not (Table 1). Sterile water inoculation (control) did not cause wilting of potatoes from 5 to 20DAI whereas all three test bacterial isolates were pathogenic to potato plants (Table 1).

Table 1. Disease incidence (DI) and disease severity (DS) over time caused by inoculation of potato seedlings with different isolates of *Ralstonia solanacearum*.

Isolate	DI %				DS (%)			
	5DAI	10DAI	15DAI	20DAI	5DAI	10DAI	15DAI	20DAI
Kinigi	10.03^{a}	27.21^{a}	87.08^{a}	98.25^{ab}	30^{a}	60^{a}	84^{a}	97.33^{ab}
Kirundo	9.51^{a}	16.71^{a}	69.05^{b}	92.61^{b}	23.33 ^{a}	44.7 ^{a}	70.67^{b}	94.67^{b}
Gikungu	1.55^{ab}	22.95^{a}	88.52^{a}	100^{a}	20^{a}	58.33^{a}	84^{a}	100^{a}
Control	0^{b}	0^{b}	0^{c}	0^{c}	0^{a}	0^{b}	0^{c}	0^{c}
P values	0.0107	0.0024	<0.0001	<0.0001	0.3811	0.0159	<0.0001	<0.0001

DAI: Days after inoculation. Values within a column followed by the same letter are not significantly different at p≤0.05.

Pathogenicity of bacteria isolated from tubers of the three potato cultivars (Kinigi, Kirundo and Gikungu) was tested based on wilting severity and disease incidence they caused to Kirundo potato seedlings under greenhouse conditions. From 5 to 10 DAI, all bacterial isolates caused wilting incidence and increased severity in potatoes which were not significantly different between them. However, from 15 to 20 days after inoculation, Gikungu and Kinigi isolates started to cause more wilting as well as an increase in severity to potatoes than Kirundo isolate. All test bacterial isolates were pathogenic to potato seedlings. Moreover, bacteria isolated from Gikungu and Kinigi were more virulent to potatoes than Kirundo isolate. Usually, the difference in virulence may be a result of inoculum from different races and biovars of *R. solanacearum*, different plant hosts, or different plant cultivars due to their genetic base as well as isolate collected from different areas (Hayward, 1991; El-Habbaa et al., 2016; Sikirou et al., 2017). In this study, bacteria were isolated from the same site (Kinigi) and the same plant host (potato) and they were also inoculated in the same host and the same cultivar (Kirundo potato cultivar). In addition, all test isolates belonged to the same biovar (biovar 3 of race 1). These may explain why similar pathogenicity rate of these isolates on one potato cultivar during some periods of the study (5 to 10 DAI) were observed. On the other hand, bacterial cells were isolated from three different potato cultivars (Kinigi, Kirundo and Gikungu). Thus, it is not surprising that the same pathogen isolated from different cultivars resulted to slight difference in the virulence they caused to potatoes during this study especially at 20 DAI.

Biovar identification

From the findings of biovar identification in the present study, all test bacterial isolates acidified all sugars (cellobiose (C), maltose (M), and lactose (L)) and alcohols (dulcitol (D), mannitol (M), and sorbitol (S)) since the medium color changed from green to yellow after four and seven days of plate incubation at 28°C (Figure 6). The ability to utilize both sugars and alcohols characterize biovar 3 of *R. solanacearum* (Lepoint and Maraite, 2002; Muthoni et al., 2012; Sikirou et al., 2017). Therefore, all the three Rwandan collected isolates causing wilting to potato seedlings belonged to *Ralstonia* biovar 3.

Furthermore, it has been reported that there is a relationship between biovars and races of this pathogen and their location (Priou et al., 2001; Muthoni et al., 2012; Sikirou et al., 2017). In this context, biovar 3 generally belongs to race 1 of *R. solanacearum,* a race which usually affects potato and other plant species such as tomato, eggplant, tobacco, chili, peanut, groundnut and several weeds in tropical lowland regions (Fock et al., 2001; Strange and Scott, 2005; Sikirou et al., 2017). However, all the three bacterial isolates were collected from Kinigi site in Musanze District, a region characterized by tropical highland conditions (Birasa et al., 1990; MINIRENA, 2013). Under such conditions, potatoes are mainly affected by *R. solanacearum* biovar 2 race 3, a race which is well adapted to cool temperatures and which affect potatoes and tomatoes (Priou et al., 2001; Muthoni et al., 2012; Popoola et al., 2015). However, the presence of biovar 3 in Kinigi site was previously confirmed by studies conducted by Butare (1987) and Lepoint and Maraite (2002). All these findings confirmed that in Rwanda *R. solanacearum* race 1 biovar 3 can also adapt to tropical highland areas and affect potatoes under these conditions. Therefore, it is concluded that all three isolates from Kinigi site (Rwanda) belonged to race 1 biovar 3 of *R. solanacearum*.

Race 1 biovar 3 is the most widely distributed type of *R. solanacearum* in the world because it has a wide range of plant hosts with potatoes included among the others (Popoola et al., 2015). In addition, potatoes are mainly propagated through vegetative planting materials, a method which favors the dissemination of the bacteria from mother tubers (Hayward, 1991). Therefore, it is not surprising to find race 1 biovar 3 of this pathogen in Musanze since long time ago it is one of the major potato growing areas and the main site of potato seed production in Rwanda (RAB, 2012; Uwamahoro et al., 2018). Thus, the occurrence may be due to the

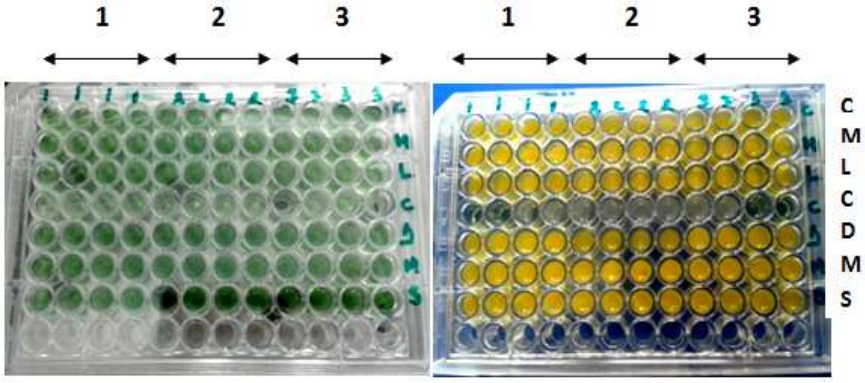

Figure 6. Biovar identification of three isolated of *R. solanacearum* strains. Green color (left) of initial basal medium changed to yellow color (right) at 4 and 7 days of incubation at 28ºC in the wells inoculated with Kinigi (1), Kirundo (2), and Gikungu (3) isolates. C: Cellobiose; M: Maltose; L: Lactose; D: Dulcitol; M: Mannitol; S: Sorbitol, Sterile distilled water (C) in 4th row of plate served as control.

introduction of this strain through potato seeds latently infected by this bacterial strain. To sustain potato production in this region, different potato genotypes should be introduced in order to identify cultivars which are high yielding and adaptable to this region with resistance to the different abiotic and biotic stresses.

Conclusions

This is the first report on a study conducted in Rwanda on pathogenicity of three bacterial wilt isolates on potato host plant. From *in vitro* experiment, all the three (Kinigi, Kirundo and Gikungu) isolates which were collected from Musanze District, Kinigi Sector showed typically cultural, morphological and biochemical traits of virulent *R. solanacearum* on both TTC and CPG agar media. In addition, from biovar test, all these test isolates belonged to *R. solanacearum* biovar 3 which is one of the biovars that belong to race 1 of this pathogen. Furthermore, *in vivo* experiment revealed that all isolates were pathogenic to potato plants. Gikungu and Kinigi isolates are more virulent to potatoes and can cause higher levels of wilting than Kirundo isolate.

Therefore, from this study, it is recommended to use Gikungu or Kinigi bacterial wilt isolates in future tests that may be conducted on *R. solanacearum*. It is also recommended to carry out other studies on biovar test or pathogenicity of *R. solanacearum* either by using a high number of bacterial wilt isolates or inocula collected from different agro-ecological zones as well as by using different hosts in order to determine whether there are other biovars and races of the pathogen that affect potato plants in Rwanda or if there are other isolates that can be more virulent to potatoes than the ones that were used in this research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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