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African Journal of Biotechnology

Full Length Research Paper

Isolation and characterization of *Ralstonia solanacearum* strains causing bacterial wilt of potato in Nakuru County of Kenya

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In Africa, cultivation of potato (*Solanum tuberosum*) represents an important food source and income generation. However, its productivity is constrained by biotic and abiotic stresses. Bacterial wilt caused by *Ralstonia solanacearum* is an important constraint to the world potato industry. In Kenya, the disease affects 77% of potato farms causing yield losses of up to 100%. Control methods are limited mainly due to the broad diversity and wide spread of its pathogen. Understanding the population structure and geographical distribution of this pathogen is an important starting point in the development of effective control strategies. In this study, *R. solanacearum* strains affecting potato cultivation in Nakuru county were successfully isolated and characterized. Kelman's triphenyl tetrazolium chloride media were used to isolate the pathogen and 20 isolates were selected based on their virulence for further characterization and confirmation of their status at the molecular level through polymerase chain reaction using 759/760 primers and sequencing of partial endoglucanase (*egl*) gene. The phylogenetic assay was done using specific primers and it was found that the phylogenetic diversity was highly heterogeneous, since all the four phylotypes of *R. solanacearum* were identified. Phylotype I was the most prevalent phylotype and represented 50% of the collection. Based on their ability to utilize sugars and alcohols, all the isolates were grouped as biovar III except 2 (Rs18 and Rs49). The aggressiveness of isolated bacteria was then evaluated using a hypersensitive reaction test on tobacco and their virulence was further confirmed on a susceptible potato variety Shangi under greenhouse conditions. All isolates elicited a reaction in tobacco with different grades. They also showed varying levels of virulence with Rs6 isolate being the most virulent. Taken together, these findings provide baseline information for improvement programs targeting host-based resistance to multiple strains causing bacterial wilt of potato in this region.

Keywords: Bacterial wilt, Biovar, hypersensitive reaction, pathogenicity, phylotype, potato, *Ralstonia solanacearum*.

INTRODUCTION

Potato (*Solanum tuberosum*), a starchy edible tuber belonging to the Solanaceae family is one of the most important food crops in the world (FAO, 2010). The nutritional value of this crop is attributed to its ability to supply high quality proteins, essential amino acids, vitamins, minerals and trace elements to the human diet

(Abong et al., 2009; Ahuja et al., 2013). The plant is ranked the fourth staple food crop after rice, wheat and corn with its average global production estimated at 380 million tonnes under production on 20 million hectares of land in 2016 (FAOSTAT, 2018). In Kenya, potato is the second most cultivated crop after maize with an annual production of about 3 million tonnes which is valued at USD 500 million (GIZ-PSDA, 2011; MoALF, 2016). This represents 8 to 15 t/ha in terms of average production relative to cultivated area which is low compared to the expected 30 to 40 t/ha under normal field conditions (Muthoni et al., 2013; Gitari et al., 2018). The low yield is mainly attributed to various challenges ranging from biotic to abiotic stresses. This includes lack of nutrients, pests and high incidences of diseases particularly, bacterial wilt disease caused by *Ralstonia solanacearum* which is still the most devastating disease of potato and the Solanaceae family as whole (Kaguongo et al., 2010). The disease has been found to occur in all the potato growing areas of the country affecting 77% of potato farms and causing yield losses of 50 to 100% (Kaguongo et al., 2010; Muthoni et al., 2012).

Several management practices have been proposed and implemented to control the disease and these include phytosanitary, cultural and chemical methods as well as breeding for resistance. However, these strategies have not been 100% effective in controlling the disease, although in locations where the pathogen is established, a combination of diverse methods have shown some promising results (Champoiseau et al., 2009). The diversity of *R. solanacearum* species, couple with its wide host-range as well as its persistence in the soil are the most significant impediments to the existing control methods. Diversity is key not only in understanding the phyto-pathological interactions between the host and pathogens but also in developing control measures. Virulence and pathogenicity (Hayward, 1991; Kinyua, 2014), phylotype (Fegan and Prior, 2005; Sagar et al., 2014) and biovar determination based on biochemical properties (Fegan and Prior, 2005) are among strategies employed in identification and characterization of *R. solanacearum* strains. Recently, phylotype have been improved to sequevar level based on the similarity of a 750-bp fragment of the endoglucanases (*egl*) gene and so far, 55 sequevars have been identified (Li et al., 2016; Liu et al., 2017).

The current study sought to isolate, characterize and define the population structure of *R. solanacearum* strains affecting potato cultivation in Nakuru County-Kenya. Here, successful recovery and identification of the pathogen from infected potato plants with further classification of the isolates into respective biovars and

phylotypes were reported. It is envisaged that this information will contribute towards an integrated management approach for better control of bacterial wilt disease, resulting in reduced losses and poverty.

MATERIALS AND METHODS

Survey site, sample collection and identification of *R. solanacearum*

Samples entailing plants presenting typical bacterial wilt symptoms (CIP, 2017) were randomly collected from ten farms in three different sub-counties in Nakuru county located within the Great Rift Valley. A total of 10 farms were surveyed for wilting plants in Kuresoi North, Njoro and Mau Narok. Five samples per farm were packaged in collection bags and transported to the Plant Transformation Laboratory at Kenyatta University for isolation and further characterization of pathogen. Prior to isolation, infected samples were first watched with tap water for 5 min, surface sterilized using 2% sodium hypochlorite (NaOCl) and rinsed three times with sterile distilled water. The pathogen isolation was done by plating 0.5 cm of surface the sterile plant tissue on Kelman's triphenyl tetrazolium chloride (TZC or TTC) medium (Kelman, 1954). The plated tissues were incubated at 28°C in an incubator and monitored until the bacteria colonies were formed. Individual distinct colony from each sample was streaked onto new TZC medium to obtain pure culture. The resulting isolates were given codes based on the collection areas and preserved in 25% (v/v) glycerol solution at -20°C to be used in subsequent experiments. Morphological characteristics were used to classify the isolated bacteria into virulent (based on milky, flat, irregular, fluidal colonies with pink or red color center and whitish margin) and avirulent strains (smaller, off-white and non-fluidal or less fluidal colonies). This was done on TTC medium containing 0.005% TTC according to Kelman (1954). The virulent isolates were selected for further analysis.

Molecular characterization of *R. solanacearum*

The isolated *R. solanacearum* were characterized using polymerase chain reaction (PCR) to validate their species, determine their phylotype and infer their evolutionary relationships by sequence analyses of the partial endoglucanase (*egl*) gene. To extract genomic DNA, bacterial cells retrieved from the glycerol stock cultures were grown on TZC agar medium and a single colony was transferred into nutrient broth. The cultures were then incubated overnight at 28°C in a shaking incubator. DNA was extracted using a DNA extraction kit (Qiagen, USA) according to the manufacturer's instructions. The DNA quality was checked through gel electrophoresis and quantified using a nanodrop spectrophotometer (Maestrogen, Taiwan). The *R. solanacearum* species was determined by using universal primer of *R. solanacearum* species: 759/760 which produces 281 bp amplicons of the species genome, a common region among *R. solanacearum* (Fegan and Prior, 2005). Identification of the phylotypes was done using a multiplex PCR based on phylotype-specific primers shown in Table 1 as described by Fegan and Prior (2005) and Sagar et al. (2014). A DNA sample was randomly selected from each resulting phylotype, to carry out a PCR targeting the endoglucanase gene

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Table 1. Primers used for species validation, phylotypes determination and endoglucanase gene sequencing.

Primer name	Sequences	Amplicon size (bp)	Tm (°C)	Remark
759R	5'-GTCGCCGTCAACTCACTTTCC-3'	280	55	Species validation
760F	5'-GTCGCCGTCAGCAATGCGGAATCG-3'			
Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144	55	Phylotype I
Nmult:21:2F	5'-AAGTTATGGACGGTGGAAGTC-3'	372	55	Phylotype II
Nmult:23:AF	5'-ATTACGAGAGCAATCGAAAGATT-3'	91	55	Phylotype III
Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3'	213	55	Phylotype IV
Nmult:22:RR	5'-TCGCTTGACCCTATAACGAGTA-3'	-	-	-
Endo-F	5'-ATGCATGCCGCTGGTCGCCGC-3'	~750	62	*egl* gene
Endo-R	5'-GCGTTGCCCGGCACGAACACC-3'			

Tm, Melting temperature.

shown in Table 1. All PCR amplifications were carried out in a 25 µl reaction mix containing: One Taq 2X Master mix with standard buffer (New England, Biolabs), 0.2 µM of each primer and 2 µl of the 5 µg/µl DNA template. Amplifications were done in an automated thermocycler (Eppendorf AG, 22331 Hamburg, Germany) using the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles comprising denaturation at 94°C for 30 s, the annealing temperatures specific to each primer for 30 s and extension at 72°C for 30 s. Final extension was done at 72°C for 5 min. The phylotype of each isolate was determined according to the size of the amplified product (Table 1) after separation on 1.5% agarose gel stained with SYBR® Green and visualized under UV light transilluminator (UVIdoc HD2, Cambridge). The PCR products for sequencing were cleaned using a PCR purification kit (Qiagen, USA) then sequenced using the forward and reverse primers at Inqaba Biotech (Inqaba, South Africa).

Sequence analysis and phylogeny

The sequences obtained from Sanger sequencing platform were retrieved from Inqaba's server. The primer sequences were removed using Vector NTI Advance (Invitrogen, USA). Resulting sequences were then used to query nucleotide databases at National Center for Biotechnology Information (NCBI, USA) using nucleotide Basic Local Alignment Search Tool (BLASTn). Four best matching results per query sequence were selected based on the highest percentage of identity and low E-values. The selected matches were then retrieved and aligned using default parameters in ClustalW's tool in MEGA7 software. Phylogenetic tree was constructed using Maximum Likelihood (ML) algorithm based on Jukes-Cantor model with 1000 bootstrap resampling of the data to test the tree topologies (Kumar et al., 2016).

Biovar determination

Isolated *R. solanacearum* were differentiated into biovars based on their ability to oxidize three disaccharides (maltose, lactose and cellobiose) and three hexose alcohols (mannitol, sorbitol and dulcitol) as previously described by Hayward (1954). Standard biovar test medium (basal medium) was prepared by adding 1.0 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1.0 g bactopeptone, 3.0 g agar and 80 mg Bromothymol blue into a final volume of 1 L of distilled water (Denny and Hayward, 2001). The pH was adjusted

to 7.0 then 10% solutions of cellobiose, lactose, maltose, dulcitol, mannitol and sorbitol were prepared separately and 1 part of each carbon solution was mixed with 9 parts of the basal medium to obtain a final concentration of 1% of the carbohydrate. The medium was autoclaved and allowed to cool before 150 µl of each preparation was dispensed into the 96 wells micro-titration plates. All isolates were inoculated into individual wells with 50 µl of bacterial suspensions adjusted to OD_{600} = 0.1 (~10^8 cfu/mL), with two replicates per isolate. The plates were incubated at 28°C and monitored daily for change in pH by a color change (Schaad et al., 2001). The experiment was repeated 3 times.

Determination of *R. solanacearum* aggressiveness using hypersensitivity reaction test

A total of 20 *R. solanacearum* isolates were determined as virulent based on Kelman (1954) method. To further analyze the aggressiveness of the 20 isolates, a hypersensitive reaction (HR) test was conducted using tobacco model plant through bacterial infiltration. Briefly, tobacco plants (*Nicotiana tabacum*) were first grown on autoclaved soil and maintained in a glasshouse at 18 to 22°C with regular watering. The inoculum was prepared by growing bacterial overnight in liquid nutrient broth medium as earlier described then pelleted by centrifugation. The concentration of cell suspension was adjusted to OD_{600} = 0.1 (~10^8 cfu/mL) using a spectrophotometer (JENWAY 6300, Dunmow, UK). Fully expanded leaves from 54 days old plant were infiltrated with a suspension of each *R. solanacearum* isolate using a sterile syringe following the injection technique described by Klement (1963). Sterile distilled water was used as a negative control while an isolate provided by CIP was also included as a positive control. Two leaves per plant from a total of 4 plants were infiltrated with each isolate and HR described as necrotic or yellowing areas in the region surrounding an infection point monitored daily for 2 weeks post infiltration. This was scored as described by Shahbaz et al. 2015). The experiment was repeated 3 times and the data were presented in a graph using graphpad prism version 6 to show the most frequent reaction induced by the identified isolates.

Pathogenicity test of *R. solanacearum* on potato

To confirm the virulence of the *R. solanacearum* isolates, pathogenicity test was performed on potato seedlings of variety Shangi by root irrigation method according to Rado et al. (2015).

This variety was chosen based on its agronomic traits and farmers preferences as it the most common potato cultivar cultivated in Nakuru County (Mwaniki et al., 2016; Gitari et al., 2018). Certified potato seeds of this cultivar were purchased from the Agricultural Development Corporation (ADC) in Molo-Kenya and sown in autoclaved potted soil. One tuber was sown per pot and emerging seedlings thinned to leave one plant in each pot. The plants were maintained in a glasshouse with natural light conditions (12 h of light and 12 h of darkness), temperatures of 24 to 28°C and a humidity of 60%. The seedlings with 4 to 6 expanded leaves were then infected with the *R. solanacearum* isolates. To prepare the inoculum, one *R. solanacearum* isolate was randomly selected from each for each surveyed areas and grown on TZC plates for 48 h at 28°C. A single colony showing virulence (fluidal, irregular and creamy white with pink at the center) was selected from each culture and transferred into a 10 mL tube containing modified Kelman media (MKM) (French and Elphinstone, 1995) then incubated at 28°C for 24 h on a shaker (180 rpm). The cultures were then pelleted by centrifugation, the bacterial cells suspended in sterile distilled water and their concentration normalized to OD_{600} = 0.1 (~10^8 cfu/mL). Plant infection was done using the root irrigation method described by Rado et al. (2015). According to the protocol, the soil layer around the stem was scooped to the side of the plant, the main root wounded by gently scratching with a sterile 1 mL tip and 10 mL of the prepared bacterial suspension poured around the base of the injured plant. The scooped soils were returned to cover the injured area and the plants maintained under the same conditions. Ten plants were then inoculated with each of the four selected isolates. Sterile distilled water was also included as a mock infection to act as a normal control and the experiment was repeated 3 times. Virulence of the isolates was assessed by analyzing bacterial wilt symptoms (CIP, 2017) on infected plants. From the first day when the wilt symptom appeared, scoring was carried out weekly for a month following Timila and Manandhar (2016) method. Plants with visible symptoms (wilted leaves) were recorded as diseased plants (Park et al., 2007). The disease incidence (DI) was calculated following the method by Xue et al. (2009) as DI (%) = [∑ (number of diseased plants in this index × disease index) / (total number of plants investigated × the highest disease index)] × 100%. The data were presented as mean of disease incidence (DI) percentage in a disease progression curve using graphpad prism version 6. The pathogen was re-isolated from diseased plants for confirmation.

RESULTS

Isolation, species validation and virulence of the pathogen

Cultural methods and media used in the current study allowed isolation of 54 isolates from the 3 sub-counties Kuresoi North, Mau Narok and Njoro sampled in Nakuru county. These isolates, suspected to be *R. solanacearum,* were subjected to various confirmatory biochemical and molecular tests to ascertain their identity. The Kelman's TZC agar differentiation test gave pink or light red color colonies with characteristic red center and whitish margin for the virulent isolates while the avirulent isolates produced smaller, off-white and non-fluidal or dry colonies on TZC medium after 48 h of incubation (Figure 1). Twenty isolates from those producing fine pink or light red color colonies were randomly selected from the sample areas for further characterization. A PCR analysis

on the 20 isolates from those producing fine pink or light red color with whitish merges colonies using 759/760 primers returned a 281 bp amplicons confirming the isolates to belong to *R. solanacearum* species as shown in the representative gel in Figure 2.

Phylotype analyses

The *R. solanacearum* strains of potato from Nakuru showed different levels of genetic diversity. The prevalence and distribution of the 4 phylotypes varied throughout the 3 major potato-growing areas in the County. The greatest diversity was found in Kuresoi North. The second highest diversity was found in Njoro, with 3 different phylotypes (I, III and IV) while all strains from Mau Narok were only identified as phylotype I. In addition, it was found that the most prevalent phylotype was phylotype I, which represented 50% of the collection (Table 2).

### Characterization of partial endoglucanase (*egl*) gene sequences

Sequencing of the partial endoglucanase (*egl*) gene allowed further confirmation of the cultures to belong to *R. solanacearum* species. The sequences have been deposited in the NCBI database awaiting accession numbers. Multiple sequence alignment and phylogenetic analysis resulted in generation of a phylogenetic tree indicating the evolutionary relationships of the current isolates with previous isolates deposited at NCBI (Figure 3). According to the Maximum Likelihood algorithm, the analyzed *R. solanacearum* clustered into 3 groups with the isolates from this study falling in groups I and III. Isolate Rs15 was found in group I, with close relation to other *R. solanacearum* and *Ralstonia syzygii* the causal agent of banana blood disease. The remaining three isolates were clustered alone in group III (Figure 3).

Differentiation of the identified *R. solanacearum* into biovars

All the 20 isolates of *R. solanacearum* with the exception of 2 (Rs18 and Rs49 whose biovars were not identified) were able to oxidize the disaccharides and sugar alcohols within 3 to 5 days although at different rates. Their ability to oxidize the substrates was confirmed by a change of the basal media color from blue-green to yellow upon incubation with bacteria isolates. Eighteen isolates which oxidized all substrates were therefore classified as belonging to biovar III. The 2 other isolates Rs18 and Rs49 were not identified due to their inability to oxidize certain substrates (Table 3). Rapid oxidation of mannitol and sorbitol were observed in relative to other
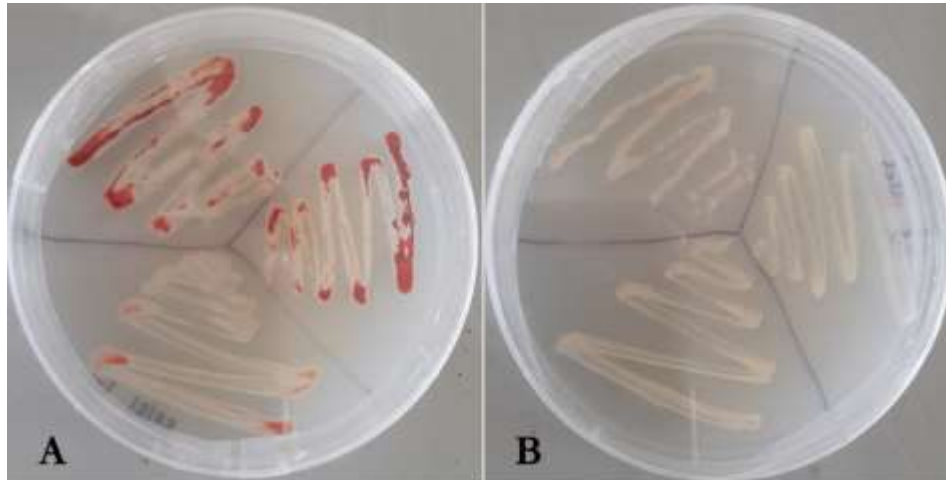
Figure 1. Appearance of *R. solanacearum* isolated on TZC medium. A, virulent colonies; B, avirulent colonies.
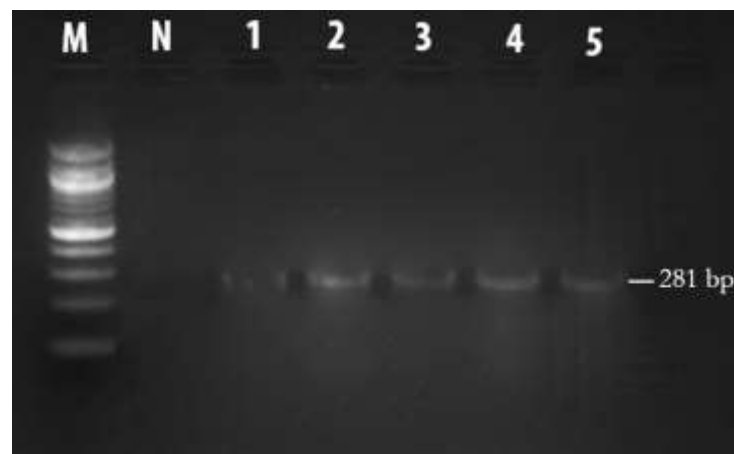
Figure 2. *R. solanacearum* species validation with 759/760 primers; M, 1kb DNA ladder; N, negative control; 1-5, bacterial isolates.

substrates. A color change in the test medium containing these substrates was observed by 2 days after culture while in dulcitol, complete color change occurred at 4 days after incubation. All the 3 disaccharides were utilized at a similar rate and resulted in complete color changes at the 4th day. Control plates with sterile distilled water did not show a color change following incubation with all bacterial isolates.

Analysis of the virulence and pathogenicity of *R. solanacearum* in plants

To assess whether there were any differences in virulence and pathogenicity of the identified *R. solanacearum* isolates, tobacco leaves were infiltrated with bacterial

inoculum of the 20 isolates and monitored development of hypersensitivity reactions. It was observed that all the isolates resulted in a hypersensitivity reaction from the infiltrated plants manifested by slight localized chlorosis followed by necrosis, although at different grades (Figure 4). Tobacco leaves infiltrated with sterile water were unaffected. Isolates Rs9A and Rs37 resulted in the highest hypersensitive response in the tobacco leaves and were therefore considered the most virulent (Table 1). On the other hand, most of the isolates were able to induce only the first grade of reaction, slight localized chlorosis (Figure 5).

Four isolates representing the four phylotypes were randomly selected following the HR assay and were used to analyze their aggressiveness on the most cultivated potato cultivar Shangi. Upon infection, disease symptoms

Table 2. Characteristics of the identified *R. solanacearum* isolates.

S/N	Isolate	Origin	Virulence	HR	Phylotypes
1	Rs1A	Muthera farm1	+	+	III
2	Rs2A	Mau Narok farm2	+	+	I
3	Rs4A2	Mau Narok farm1	+	+	I
4	Rs6	Kuresoi North farm6	+	++	IV
5	Rs8	Mau Narok farm2	+	+	I
6	Rs9A	Kuresoi North farm2	+	+++	IV
7	Rs13	Muthera farm2	+	+	I
8	Rs13B	Muthera farm2	+	++	I
9	Rs15	Kuresoi North farm3	+	++	II
10	Rs18	Kuresoi North farm6	+	+	II
11	Rs19	Muthera farm1	+	++	III
12	Rs21	Kuresoi North farm5	+	+	III
13	Rs26	Mau Narok farm2	+	++	I
14	Rs34	Muthera farm1	+	+	IV
15	Rs35	Muthera farm1	+	+	I
16	Rs36	Kuresoi north farm1	+	++	I
17	Rs37	Kuresoi north farm2	+	+++	IV
18	Rs49	Mau Narok farm2	+	+	I
19	Rs56	Mau Narok farm4	+	++	I
20	Rs57	Muthera farm1	+	++	III
21	Reference	CIP, Kenya	+	++	IV

HR positive score: slight localized chlorosis (+) followed by necrosis (++) and collapse of whole leaf (+++); in virulence test: + denotes a positive reaction showing colonies with fine characteristic of virulent strains of *R. solanacearum* as described by Kinuya (2014).
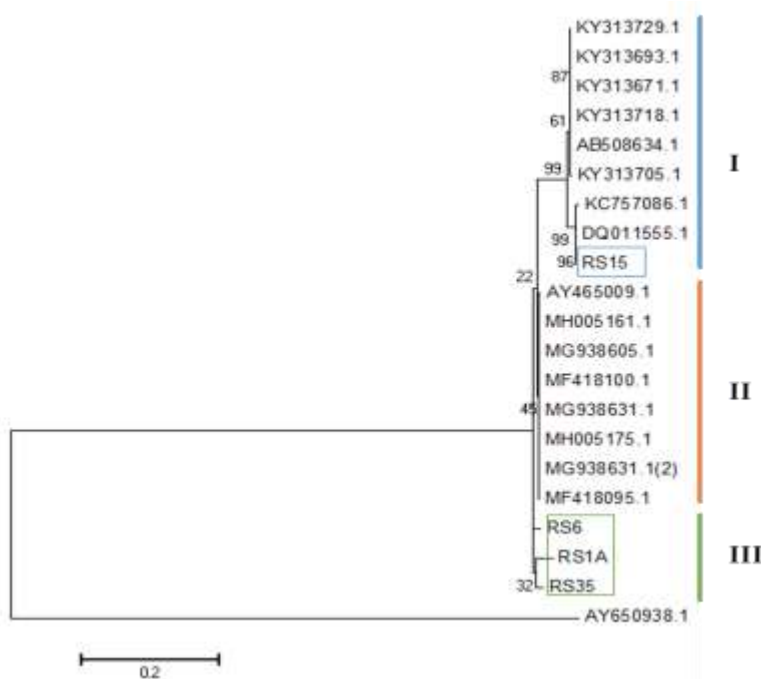
Figure 3. Evolutionary relationships between the identified *R. solanacearum* strains and other known sequences from NCBI. The phylogenetic tree was generated using the maximum Likelihood algorithm in MEGA 7 following a multiple sequence alignment.

Table 3. Biovar differentiation of the identified *R. solanacearum* isolates.

| Isolate	Cello biose	Maltose	Lactose	Mannitol	Sorbitol	Dulcitol	Control	Biovar
Rs1A	+	+	+	+	+	+	-	III
Rs2A	+	+	+	+	+	+	-	III
Rs4A2	+	+	+	+	+	+	-	III
Rs6	+	+	+	+	+	+	-	III
Rs8	+	+	+	+	+	+	-	III
Rs9A	+	+	+	+	+	+	-	III
Rs13	+	+	+	+	+	+	-	III
Rs13B	+	+	+	+	+	+	-	III
Rs15	+	+	+	+	+	+	-	III
Rs18	+	+	-	+	+	-	-	*
Rs19	+	+	+	+	+	+	-	III
Rs21	+	+	+	+	+	+	-	III
Rs26	+	+	+	+	+	+	-	III
Rs34	+	+	+	+	+	+	-	III
Rs35	+	+	+	+	+	+	-	III
Rs36	+	+	+	+	+	+	-	III
Rs37	+	+	+	+	+	+	-	III
Rs49	-	-	+	+	+	-	-	*
Rs56	+	+	+	+	+	+	-	III
Rs57	+	+	+	+	+	+	-	III
Reference	+	+	+	+	+	+	-	III

*Could not be identified.

Figure 4. Hypersensitivity responses induced on tobacco plants by tested isolates. A. Original leave; B, no reaction with sterile distilled water; C, slight localized chlorosis (+); D, followed by necrosis (++); E, collapse of whole leaf (+++).

characterized by wilting, yellowing of leaves and black streak on the stem were observed from day 8 following infection (Figure 6). The disease progression was monitored weekly for one month and the observed disease incidence (DI) ranged from 0 to 40% with Rs6 isolate (from Kuresoi North) being the fastest and most virulent followed by Rs15 also from the same region. The isolates from Mau Narok and Njoro (Rs1A and Rs35) were weakly aggressive and Rs35 showed no wilt symptoms but resulted in yellowing of leaves and

development of black streak on the stem, compared to the control where none of these symptoms were observed (Figure 7).

DISCUSSION

The findings of this study show successful isolation and characterization of *R. solanacearum* causing bacterial wilt of potato in Nakuru county of Kenya. Cultural, biochemical
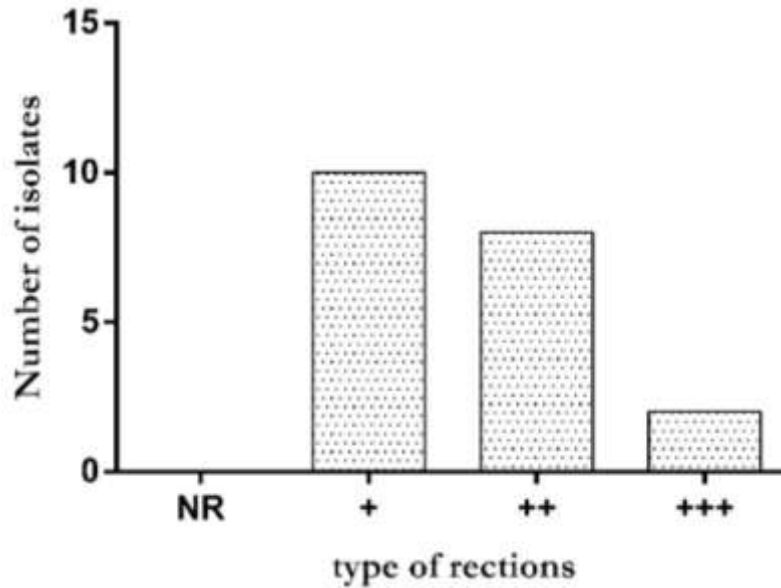
Figure 5. Different type or grade of reaction induced by the isolates and their frequency.
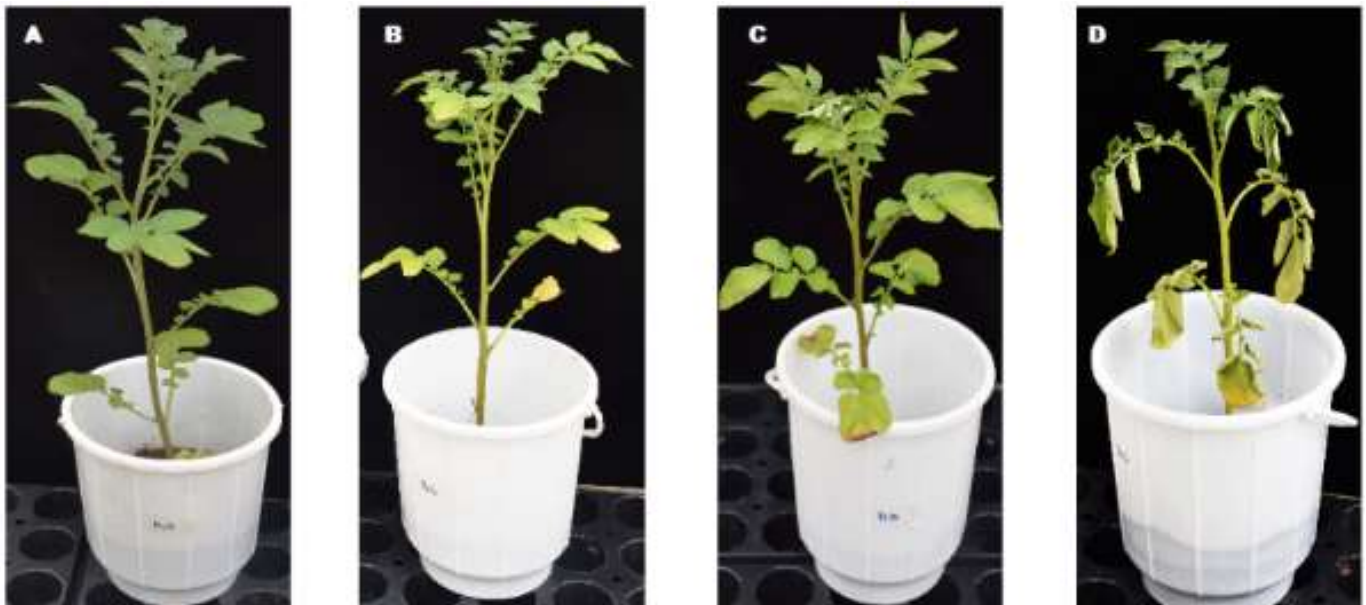

Figure 6. Different types of symptoms induced on potatoes (cv. Shangi) by the tested *R. solanacearum* strains. A. No visible symptoms (control with H_2O); B, Yellowing or chlorotic spots on the leaves; C. Black streak on the stem and wilting of the upper leaves; D, Wilting of all leaves and plant death.

and molecular methods were used to characterize the isolates into biovars, phylotypes and further determine their evolutionary relationship with other known *R. solanacearum* species all over the world. TZC medium allowed successful isolation of *R. solanacearum* as revealed by the different pigmentation. Apart from

selective isolation, this medium has also been implicated in differentiating virulent from avirulent strains with Kelman (1954) reporting that virulent colonies appear white with pink or light red centers and non-virulent colonies appear as small off-white colonies. On this medium, typical virulent bacterial colonies (fluidal,
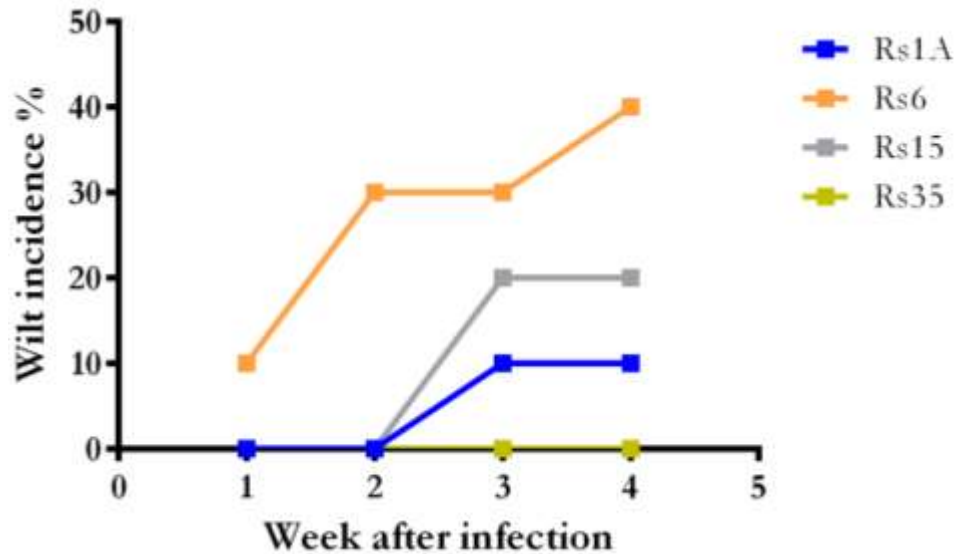
Figure 7. Tracking disease progression on potato cv. Shangi using four *R. solanacearum* isolates

irregular in shape and white with pink or light red centers) were obtained as also report by Champoiseau and Momol (2008)) and Rahman et al. (2010). The isolates were then confirmed to be *R. solanacearum* species, in line with the work by Fegan and Prior (2005) where they also reported the amplification of 281 bp of the common region of the species genome with 759/760 primers.

The isolated *R. solanacearum* was further characterized into biovars based on their ability to utilize sugars and/or alcohols. Differentiation of *R. solanacearum* into biovars based on the utilization of carbohydrates has been previously reported by Hayward (1964). It was found that all isolates from this study successfully oxidized the disaccharides and hexose alcohols and this confirmed their classification as biovar III, relative to what was established by Denny and Hayward (2001). The successful isolation of *R. solanacearum* which all belong to biovar III confirmed their ability to adapt to several conditions (Denny, 2007).

Moreover, the high virulence of biovar III strains in the region can be justified by their wide host range and compatibility with number of environmental factors favorable for disease appearance such as temperature, rainfall, soil type, inoculum potential, and other soil biological factors such as wilt complexes formed among nematodes (*Meloioigyne* species) fungi (*Fusarium* species) and *R. solanacearum* (Shahbaz et al., 2015).

Cultural and biochemical methods in bacterial identification is often supported by molecular characterization using known sequences to further affirm the categories to which each isolate belong to. In the current study, the phylotypes of each isolate were determined and it was found that all 4 phylotypes of *R. solanacearum* (I, II, III and IV) were present in the region.

Even though, all phylotypes were found from the sampled regions, their distributions were not even. For instance, all the 4 phylotypes were recovered in Kuresoi North, 3 in Njoro and phylotype I was the only phylotype isolated in Mau Narok. The uneven distribution of this pathogen could be due to differences in adaptation to climatic conditions and the competitive fitness advantage of *R. solanacearum* as earlier reported by Huerta et al. (2015). Phylotype I, exhibited the highest incidence than the others phylotypes. This can be explained by its capacity to infect a wide range of hosts including herbaceous and woody plants (Hayward, 1994). Furthermore, phylotype I is known to be distributed worldwide (Hayward, 1991) and it is reported to be highly recombinogenic (Coupat et al., 2008; Wicker et al., 2012). Several studies have also reported prevalence and distribution of different phylotypes in other African countries. For instance, in Cameroon and Ivory Coast, phylotypes I, II and III have been reported (Mahbou et al., 2009; N'Guessan et al., 2012) while in Ethiopia phylotypes I and II have been identified (Lemessa and Zeller, 2007). Phylotype I was also previously reported in Madagascar and eastern African countries bordering the Indian Ocean including Kenya and South Africa (Wicker et al., 2012; Ravelomanantsoa et al., 2016; Carstensen et al., 2017). This is the first report of phylotype IV in Kenya which could have been introduced in the country through imported potato seed with latent infection (Kaguongo et al., 2010).

Sequencing of the endoglucanase gene from the isolated *R. solanacearum* confirmed the identity of these bacteria and allowed deciphering of their evolutionary relatedness with other known R. solanacearum species. This gene has previously been used for sequevar

determination (Li et al., 2016; Liu et al., 2017), evolutionary dynamics to reveal genetic relationships between *R. solanacearum* species complexes (RSSC), phylogenetic and statistical analysis of housekeeping, virulence-related and pathogenicity-related genes (Fegan and Prior, 2005; Castillo and Greenberg, 2007).

All *R. solanacearum* isolates elicited a hypersensitive response from infiltrated tobacco leaves which was a good indicator of their potential virulence. Hypersensitive reaction is a defense mechanism used by plants to prevent the spread of pathogen infection to non-infected parts. It is associated with plant resistance and characterized by rapid and programmed cell death localized in the region surrounding an infected region (Nimchuk et al., 2003). Reports have indicated that most pathogenic bacteria induce hypersensitivity in leaves of tobacco or other non-host plants and this is often used as a prescreen technique in virulence assays (Poussier et al., 2003; Yabuuchi et al., 2006). In *R. solanacearum*, the hypersensitivity reaction and pathogenicity genes or *hrp* genes have been implicated in controlling induction of both disease development and hypersensitive reactions with *hrp* mutants unable to induce symptoms in susceptible host plants (Boucher et al., 2001). The ability of all isolates under this study to induce hypersensitive reaction (HR) could be attributed to presence of the *hrp* genes in their genome as reported by Boucher et al. (2001).

The virulence of these isolates was determined through pathogenicity tests on a susceptible potato cultivar Shangi. Here, plants were infected with different isolates with a uniform concentration of the pathogen and maintained under the same conditions then evaluated for disease occurrence and severity, as previously reported by Timila and Manandhar (2016). It was observed that the isolates caused disease symptoms on potato plants albeit at different rates. The nature of these symptoms also varied from those observed under field conditions as also reported by Huerta et al. (2015). This could be due to the fact that *R. solanacearum* pathogenicity is distinctly regulated in early or late stages of infection in response to environmental conditions such as soil, humidity, temperature and texture as well as bacterial population densities (Schell, 2000; Hikichi et al., 2007). It is important to note that *R. solanacearum*-host interaction occurs through three main stages including root colonization, cortical infection and xylem penetration. These stages are affected by plant structure and metabolism; and these could have played a role in the observed phenotypes upon infection (Vasse et al., 2005).

Conclusion

R. solanacearum strains affecting potato in Nakuru county-Kenya were successfully isolated and characterized using phylogenetic and pathogenetic analyses. It was found that the pathogen is highly diverse with several phylotyes and variable distribution. Phylotype I was found as the most predominant phylotype and widely distributed in the region. Therefore, it should be considered in development of control strategies such as grafting for resistance or propagation programs. The results also demonstrated that biovar III strains have adapted themselves to the more diverse environment of Nakuru than other biovar strains showing severe reaction on tobacco and variable virulence on potato plants. These findings provide vital information on the *R. solanacearum* strains in this region as well as their associated virulence and distribution which form a basis for breeding programs for potato bacterial wilt resistance and development of control strategies with special emphasis on the improvement of pathogen-targeted and geographically-targeted management practices.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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