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Diversity of *Ralstonia solanacerum* Strains in Solanaceous Crops Production Regions of Central Kenya

E. K. Kago[1*], Z. M. Kinyua[2], J. M. Maingi[1] and P. O. Okemo[1]

[1]Department of Microbiology, Kenyatta University, P.O.Box 43844-00100, Nairobi, Kenya.
[2]Plant Pathology Section, KALRO-NARL Kabete, P.O.Box 14733 – 00800, Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration between all authors. Author EKK did the sample collection, isolated and performed the biochemical analysis of biovars, analyzed data and prepared the first draft. Author ZMK provided the working protocols for biovar analysis and guided (Supervised) the activity. Authors JMM and POO provided general guidance on the experiment and edited the manuscript. All authors read and approved the manuscript.

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ABSTRACT

Aims: Bacterial wilt disease is a very serious yield limiting problem in crops in the solanaceae family grown in regions of Kenya. The very much encouraged field sanitation and use of clean planting materials have been inadequate in combating this challenge. This has seen increased need for understanding the genetic and biochemical diversity of *Rastonia solanacerum* stains common to these areas as a basis for better strategies in their control.
Methodology: A field survey accompanied by samples collection was conducted covering: Nyeri, Nyahururu, Kirinyaga, Kiambu, Nakuru Murang'a and Embu Counties exclusively for solanaceae crop farms. Purposeful sampling was employed.
Results: A total of 160 samples of bacterial wilt symptomatic plants were collected, 120 isolates were confirmed as *R. solanacearum* using biochemical tests. They were further subjected to biovar tests using sugars and alcohols, (Cellobiose, Lactose, Maltose, D-Ribose, Salacin, Dulcitol,

**Corresponding author: E-mail: kagoelizabeth96@gmail.com;*

Mannitol, Trehalose and Sorbitol). Dextrose and Salacin were used as positive controls while sterile distilled water was used as negative control respectively. The results enabled mapping of *R. solanacearum* biovar in these regions. Biovar 2 was the most prevalent in the study area with biovar 2T showing high prevalence in both highlands and lowlands; biovar 2A was common in lowlands. Other biovars identified were 1 and 3.

Conclusion: This survey findings indicated that *R. solanacerum*, biovars present in Kenyan Highlands and Lowlands included biovars 1, 2 and 3. These biovars are highly pathogenic on solanaceae crops throughout the world. The findings of the present study will be useful for designing the study of the population structures of *R. solanacearum* using the molecular approaches with special emphasis on its integrated management.

Keywords: Bacterial wilt; Kenya; biovars; control.

ABBREVIATIONS

0℃ : Degrees celsius
CPG : Casamino acid-Peptone-Glucose (CPG) medium
Cel : Cellobiose
D-Rib : D-ribose
Dulci : Dulcitol
EEPPO : European and Mediterranean Plant Protection Organization
Malt : Maltose
Mant : Manitol
Lact : Lactose
L-Trypt : L (-)Tryptophan
Sorb : Sorbitol
Tre: D(+): Trehalose
TZC : Triphenytetrazolium chloride (TTC or TZC) medium.

1. INTRODUCTION

Tomatoes, capsicum and potatoes are among the most important vegetables in Kenya. Their increased production and supply within the country is very important for food and nutritional security. These crops are majorly affected by diseases and pests. With scenarios of increased resistance of the pests to common control measures arising, there is need for more research for betterment of their management. Amidst plant diseases, *Ralstonia* spp is a phytopathogenic bacterium causing great losses in *Solanaceae* crops production in Kenya. *R. solanacearum* is an aerobic, gram negative, non-spore forming non-capsulated rod shaped bacterium and nitrate-reducing, ammonia-forming, aerobic, rod-shaped bacterium [1]. The bacterium, is among world most destructive pathogens, [2] it was first reported in South America and Asia in1880's, [3] and is now spread worldwide, [4]. The pathogen is highly heterogeneous with many distinct strains differing in natural host range, and geographic distribution, genetic and biochemical characteristics. The host range of the bacterium is expansive comprising of hundreds of plant species, [5]. Important cultivated crops affected include: *Capsicum annum* (sweet pepper), *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Capsicum* spp (Chilies), *Solanum melongena* (Eggplant), *Musa spp* (banana) *Arachis hypogaea* (groundnut) and *Nicotiana tabacum* (tobacco), [6]. Ginger, Sunflower, cassava, olive, and eucalyptus are as well susceptible. Many asymptomatic weeds harbor the bacteria such as some ecotypes of *Arabidopsis thaliana*. The common symptoms range from inhibited growth, poor leaf quality in tobacco, deterioration of leaves, vascular damage, browning and necrosis of tubers (potato) [7-8].

R. solanacearum is a species complex that has been classified into five races based on host range [9] and into five biovars based on biochemical properties [10]. There are variations in *R. solanacerum* strains in term of host relationships, phenotypic properties and phylogeny, this accompanied by rapid adaptation and mutations are key in their pathogenicity, influencing disease development and virulence factors of the strains [11]. *R. solanacearum* races and biovar are now associated with specific hosts; Race 1 biovar 1, race1 biovar 3 and race1 biovar 2; common to Asia, Africa, Australia, North America and south America attacks: ginger, *capsicum*, olive, peanut, tobacco among other *Solanum* spp plants. Race 2 biovar 1, common to Asia, Caribbean Central America, South America and Hawaii prefer *Musa* spp (plantain and banana), peanut, tomato and some *Heliconia* spp. Race3 biovar 2, is found worldwide (except Canada and United States), affecting *Solanum spp* and *Pelargonium* spp. Race 4 biovar 3 and race 4 biovar 4 are common in Australia, India, Asia, and Hawaii, attacking ginger. Race 5 biovar

5 common in China attacks, *Mura spp*. The pathogen has been further categorized into phylotypes; phylotype i (biovars, 3, 4 and 5), phylotype ii (biovars 1 and 2-both 2A and 2T), phylotype iii (biovars 1 and 2) and Phylotype iv (biovars 1, 2A and 2T), [12-14].

Rastonia spp, can survive in soil and infected plant debris over long periods [15]. This bacterium may also survive by colonizing the rhizospheres of non-host plants [16]. Elevated temperatures (30-35℃) are favorable for bacterial wilt disease, whereas soil temperatures below 20℃ are not suitable [8]. The disease prevails in diverse soil types [6]. During planting season the bacteria may be introduced into the field through contaminated planting materials, contaminated implements and old plant debris (diseased debris). Spreading of the bacteria from one plant to another is increased by contaminated irrigation water [17-18]. The objective of the study was to establish the diversity of *Ralstonia solanacerum* strains in solanaceous crops production regions of central Kenya.

2. MATERIALS AND METHODS

2.1 Collection of Diseased Samples

A total of 160 diseased solanaceae crops (potato, tomato, *capsicum* and black nightshade) were collected from highlands and lowlands of Nakuru, Nyandarua, Nyeri, Kirinyaga, Murang'a, Kiambu and Embu Counties. The diseased solanaceae crops were subjected to bacterial wilt simple bacterial streaming test and later cultured in the laboratory where they were confirmed as pathogenic *Ralstonia* spp by development of virulent colonies (fluidal, irregularly-shaped, white colonies with pink centers) 36 hours incubation on TZC media. This procedure was further supplemented with biochemical analysis for biovar determination.

2.2 Biovar Testing

2.2.1 Inoculum preparation

The *Ralstonia solanacearum* bacterial isolates were confirmed by growing the bacteria in SMSA Media and later multiplying it in CPG medium for 48 hrs at 28℃. A loop-full of bacteria was taken and mixed with 1 ml sterile water in a 2.0 ml eppendorf tube and shaken thoroughly to form a uniform suspension, [19].

2.2.2 Basal medium preparation

Media preparation for biovar testing was done according to standards by the international plant diagnostic network (IPDN). Ammonium dihydrogen phosphate $NH_4H_2PO_4$) 0.35 g, (Potassium hydroxide (KOH) 0.07 g, Bromothymol blue 0.0105 g, Magnesium sulphate ($MgSO_4$. $7H_20$) 0.07 g, Peptone 0.35 g, water 350 ml and Agar 1.05 g were dissolved and autoclaved at 121℃ for 18 minutes. The medium was left to cool to 60℃ and amended with sugars and alcohols. Ten ml of each prepared sugars/alcohols was added to 90 ml of the prepared basal medium. Cellobiose (Cel), Lactose (Lac), Maltose (Malt), D-Ribose(D-Rib), Salacin(Sal), Dulcitol(Dul), Mannito(Man), Sorbitol (Sorb), L-Tryptophan(L-Tryp), Dextrose (Dex), Trehalose (Tre) were used. Sterile distilled water was used as negative control while salacin and dextrose were used as a positive control [20].

2.2.3 Biovar testing procedure

The isolates of *R. solanacearum* were differentiated into biovars based on their ability to utilize disaccharides (sucrose, lactose, maltose) and sugar alcohols (manitol, sorbitol and dulcitol) as described previously by [9-10]. The biovars were determined in the mineral medium ($NH_4H_2PO_4$ 1.0 g, KCl 0.2 g, MgSO4·7H2O 0.2 g, Difco bacto peptone 1.0 g, Agar 3.0 g and Bromothymol blue 80.0 mg per litre) containing 1% sugar. About 150 µl of the melted medium was dispensed into the wells of microtitre plate. Inoculums for each group of isolate was prepared by several loop full of bacteria from 24-48 hours old cultures to distilled water to make suspension containing about 108 CFU/ml. Then 20 µl of bacterial suspension was added to the wells of microtitre plate incubated at 28℃. The plates were then examined after 3-5 days of inoculation for changing pH which was indicated by the change of colour. The colour of the medium changed to yellow for positive results while those that remained blue-green colour exhibited negative results (Plate 1). Descriptive statistics were carried out to generate percentages of biovars on crops and study areas and graphs used to present the results.

2.2.4 Biovar diversity determination

R. solanacerum biovars diversity in the study areas was determined based on sugar utilization using a number of diversity indices: Dominance_

DShannon_H, Berger-Parker, Simpson_1-D, Brillouin, Margalef, Fisher_alpha, Evenness_ e^H/S, Menhinick, Equitability_J and Chao-1. This enabled development of diversity profile. A dendogram was drawn grouping the biovars isolated into clusters. Biochemical diversity indices were calculated using PAST software version 3. The diversity profiles were drawn based on Renyi index which is a generalization of Shannon's informational measure of diversity extrapolated to particular moments of the same function with a scale parameter (alpha = 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, infinity using PAST software version 3.Dendrograms and PCA for biochemical diversity were drawn using PAST software version 3.

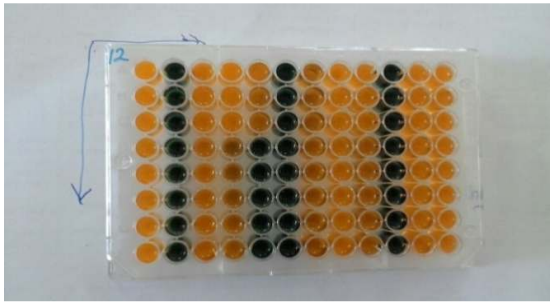

Plate 1. A 96 well plate displaying positive (yellow) and negative (blue-green) results
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3. RESULTS AND DISCUSSION

3.1 Biovars Isolated on Solanacea Crops and Variability in Study Region

Diseased crop samples collected from Nakuru, Murang'a, Nyeri, Kirinyaga, Embu, Kiambu and Nyandarua counties comprised of potato, tomato, capsicum and black nightshade. These diseased crop samples showing symptoms of bacterial wilt (stunted and vascular damage) were subjected to a series of tests to confirm the causal organisms of the disease. These proved true for *Ralstonia solanacearum.* Biochemical analyses of the bacterium indicated three biovars: biovar 2 (88.3%), biovar 1 (6.7%), and biovar 3 (5.0%), (Table 1).

Among these biovars, the predominant biovar 2 was highly exhibited on potato (55.80 %) and tomato (29.20%) and to a lower percentage on black nightshade (1.70%) and capsicum (1.70%). This biovar had been noted to cause huge losses in potato and tomato farming. There has been strict control at the market, to prevent continued spread of these bacteria to other parts, with EPPO, imposing strict measures preventing marketing of Solanacea crop products from regions where the pathogen is suspect to be present [10]. Currently there is a big challenge in managing *R. solanacerum* race 3 biovar 2 [20], since the pathogen is known to spread over long distances through potato tubers and planting materials, and some of the suggested control measures are inclusive of utilization of high quality-pathogen free irrigation water, use of certified planting materials and strict checks of the presence of the pathogen, especially at seed production units/ nurseries [18].

Biovar 1 was common on potato (5.00%) and Capsicum (1.70%), while biovar 3 was only present in isolates from potato (4.20%) and tomato (0.80%).Biovar 1 was more from samples from lowlands and less from isolates obtained from highlands. Biovar 2 was prevalent in isolates from both highlands and lowlands; biovar 3 was common to isolates from highlands and less common in isolates from lowlands (Fig. 1).

Among the three *R. solanacearum* biovars (biovar 1, 2 and 3) isolated from the Kenyan highlands and lowlands, biovar 2 was the most common, and was predominant on potatoes and capsicum. Many cases of bacterial wilt disease in Central Kenya region of potato production is likely to be due to race 3 biovar 2. This pathogen had severally been credited to huge losses in potatoes and tomatoes in Kenya. Biovar 1 was found on both potatoes and capsicum in lowlands, but on potatoes only in highlands. Biovar 2 was found attacking black nightshade, capsicum, potato and tomato in highlands, and only affecting potatoes and tomatoes in lowlands. Biovar 3 was found attacking tomatoes in lowlands and potatoes in highlands. Biovar 1 had been identified as causal organism of moko disease in bananas, brown rot in potato, it was also reported to affect tomato. Biovar 3 is pathogenic to eggplant, pepper and tomato [21-23].

Isolates from diseased samples obtained from Embu County (potato and tomato) tested positive from biovar 2 only, this was similar to isolates from Murang'a (potato, tomato and black nightshade), Nakuru (potato), Nyandarua (potato and tomato) and Nyeri (potato, tomato and capsicum). Kiambu (potato, tomato and *capsicum*) and Kirinyaga (Potato and tomato) counties' isolates had the most diverse

composition of *R. solanacearum* biovars; biovars 1, 2 and 3, (Fig. 2).

Trytophan, D-ribose and Trehalose were used to further differentiae biovar 2 into 2A and 2T. Among the biovar 2 isolated, a larger percentage was biovar 2T (85.90%) with biovar 2A (14.10%) being relatively low. Biovar 2T was prevalent in highlands (46.20%) and lowlands (39.60%), with biovar 2A appearing prevalent in highlands (13.20%), and less prevalent in lowlands (0.90%), (Table 2).

Isolates from all the seven study counties (Embu, Kiambu, Kirinyaga, Murang'a, Nakuru, Nyandarua and Nyeri), tested positive for biovar 2T. Biovar 2A was only present in isolates from four of the seven study counties (Kiambu, Murang'a, Nakuru and Nyeri), (Fig. 3).

Table 1. Bacterial wilt biovars obtained from diseased samples from Kenyan highlands and lowlands

Biovar type	Biochemical test	Test crop	% Biovar isolate	% Biovar types (1,2,3)
1	Cel-, Lact-, Malt- & Dulci-, Mant-, Sorb.-	Potato	5.00	6.7
		Tomato	0	
		Capsicum	1.70	
2	Cel+, Lact+, Malt+ & Dulci-, Mant-, Sorb.-	Potato	55.80	88.3
		Tomato	29.20	
		Capsicum	1.70	
		Black nightshade	1.70	
3	Cel+, Lact+, Malt+ & Dulci+, Mant+, Sorb.+	Potato	4.20	5.0
		Tomato	0.80	
		Capsicum	0	

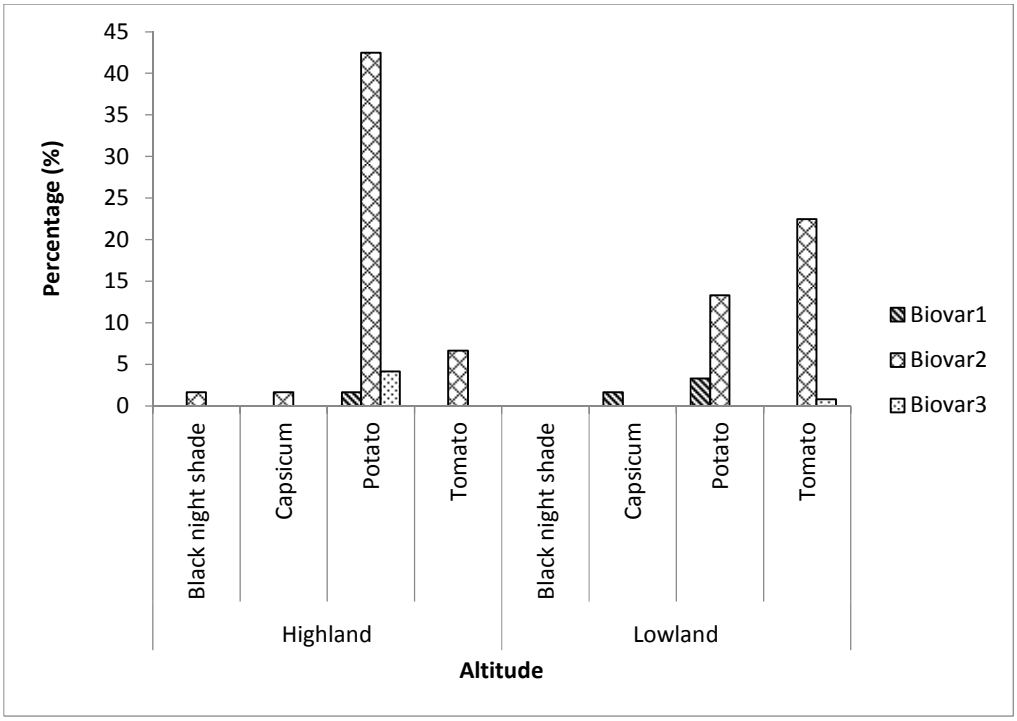

Fig. 1. *R.solanacearum* biovars isolated from black nightshade, *capsicum*, potato and tomato, for samples from both highlands and lowlands

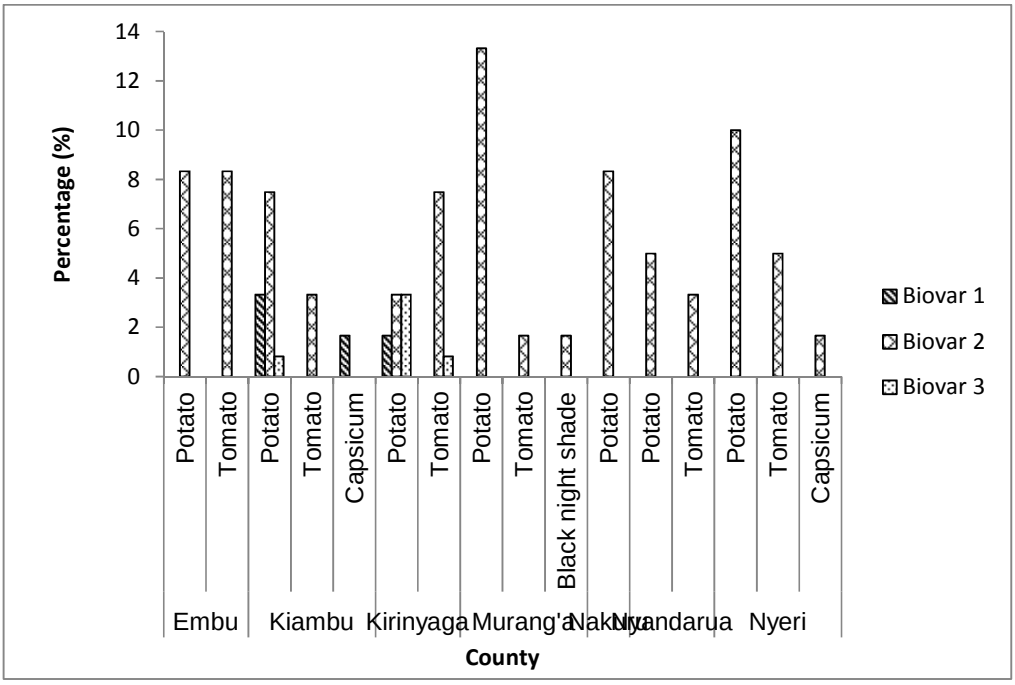

Fig. 2. Counties, solanaceae crops grown and biovars isolated

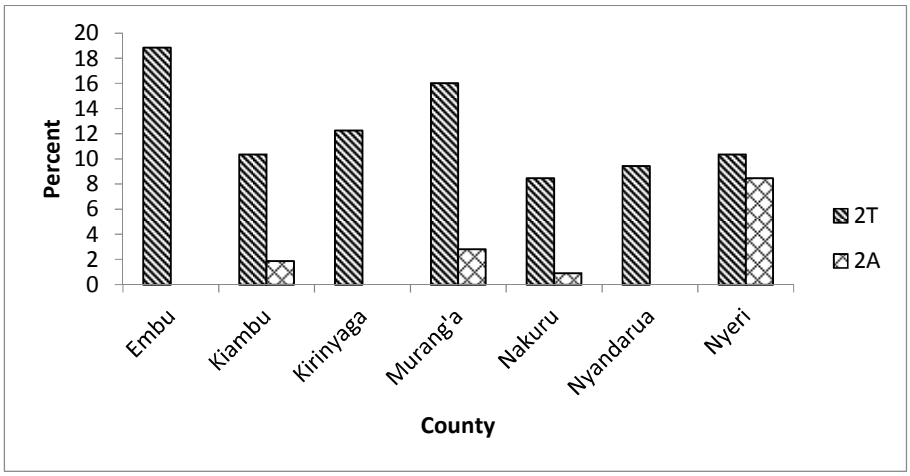

Fig. 3. *R. solanacearum* biovars 2A and 2T as isolated from samples collected from the seven study counties

Table 2. *R. solanacearum* biovars 2A and 2T zonations in Kenyan highlands and lowalands

Biovar type	Biochemical test	Zonation	% Biovar isolate	%Biovar (2A/2T)
2A	Cel+, Lact+, Malt+, L(-) Trypt-, D-Rib-, Tre- &Dulci-, Mant-, Sorb.-,	Lowland	0.90	{14.10}
		Highland	13.20	
2T	Cel+, Lact+, Malt+ L(-) Trypt+, D-Rib+, Tre+ &Dulci-, Mant-, Sorb.-	Lowland	39.60	{85.90}
		Highland	46.20	

Among the solanaceae crops used in the study, biovar 1 was predominant on capsicum (1.70%) and potato (5.00%). Biovar 2 was found on capsicum (1.70%), black nightshade (1.70%), potato (55.80%) and tomato (29.20%).Biovar 3 was found on potato (4.20%) and tomato (0.80%) only (Fig. 4).

In the highlands and lowlands biovar 2 was the most prevalent found on black nightshade, capsicum, tomato and potato in highlands, and found on potato and tomatoes in the lowlands.

The high diversity of *R. solanacerum*, is likely to confer survival in varied ecological conditions a seen in the isolates.

The results showing biovar 1 attacking both capsicum and potatoes in lowlands indicates higher virulence of the pathogen to solanaceae crops in the warm conditions, this is in line with the work by Meng et al., [24], who described biovar 1 as the most common in warmer areas. In highlands that are presumed relatively cooler, biovar 1 could only be obtained from potato derived isolates. The study found *R. solanacearum* biovar 2 to be significantly common in Kenyan highlands and lowlands. These findings are in line with work by other researchers who had singled out biovar 2 (race 3 biovar 2) as the one adapted to diverse ecological conditions. This biovar had been isolated in Cameroon's potato and tomato growing regions comprising of both highlands and lowlands, [23,24]. The biovar is exceptionally adapted to cold conditions. Milling noted high survival of biovar 2 in tubers spanning 4 months at low temperatures of about 5°C [25]. It was further discovered that this *R. solanacerum* biovar could retain viability in infected geranium debris for periods exceeding 6 months in very harsh temperature conditions of -20℃, [24]. The adaptations of biovar 2 are not unique to cold conditions, but also to lowlands, as Milling reported high virulence of biovar 2 at temperature of 20°C [23]. The same biovar had been majorly implicated in bacterial wilt disease at an average temperatures of 29.5℃ [22].

Biovar 3 of *R. solanacerum* isolated was significantly present in highlands and lower in lowlands, this contradicts earlier reports by Prior et al, who had described this biovar as one for the warmer conditions. The likely explanation for this may be linked to the possibility of occurrence in latent infections without causing disease. But it had initially been reported that biovar 3 could prevail in diverse soil pH, temperatures and be able to infect tomato, sweet pepper and eggplants in such conditions, [23].

Pathogenecity of *R. solanacerium* biovars to solanaceae family was observed to vary among the study crops (Potato, tomato, black nightshade and capsicum). Biovars 1 occurrence on potato and tomato is in line with similar observations by Milling et al. [26] and Zulperi et al. [21], who found this biovar highly virulent to tomato with low pathogenicity to potato.

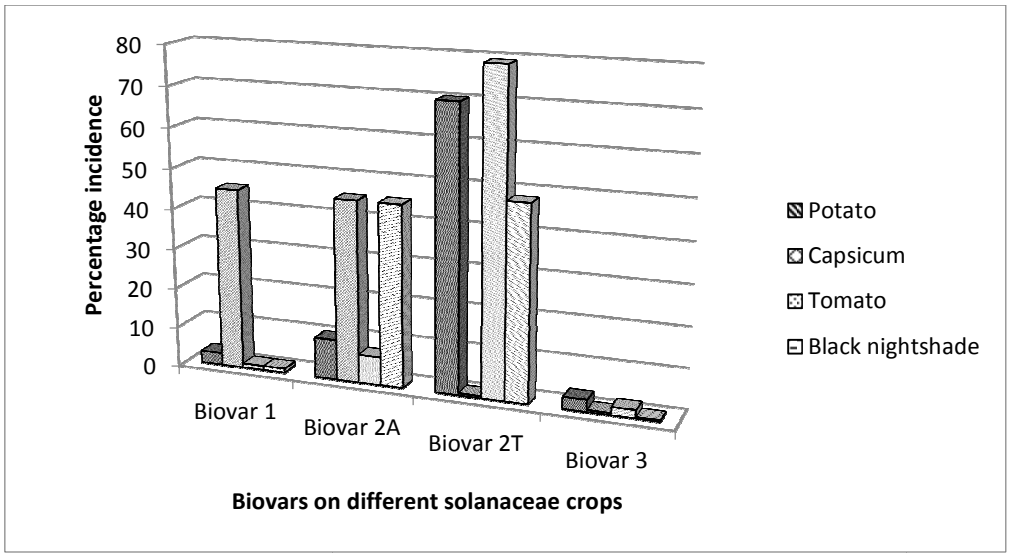

Fig. 4. *R. solanacearum* biovars 1, 2 and 3 Isolated from solanaceae crops

According to the current study, Biovar 2 (2A and 2T), was the most common biovar affecting all the study crops (tomato, *capsicum*, potato and black nightshade). This is in line with the earlier studies that had implicated this biovars on potato, tomato and many other solanaceae crops in Cameroon, Kenya and east Africa at large [27].

R. solanacearum biovar 2 has thus been detected in the highlands of the tropics as well as in several (sub) tropical and some temperate areas throughout the world [28]. This study identified Biovar 3 incidence on tomato and potato. This corresponds to knowledge of its pathogenicity to affect tomato, eggplant and capsicum, [20,25].

3.2 Biovar Diversity in the Study Region

Using varied diversity indices, the study areas varied in diversity of *Ralstonia solanacerum* isolated. Kirinyaga highland (KRH), Kiambu highland (KBH) and Kiambu lowlands exhibited the highest diversity in comparison to all the other areas. Murang'a highland (MRH), Murang'a lowland (MRL), Nyandarua highland (NYRH), Nakuru highland (NKRH), Nyeri highland (NYEH), Nyeri lowland (NYEL), Kirinyaga lowland (KRL), Embu highland (EBH) and Embu lowland (EBL) exhibited the low diversity as shown by Fig. 5.

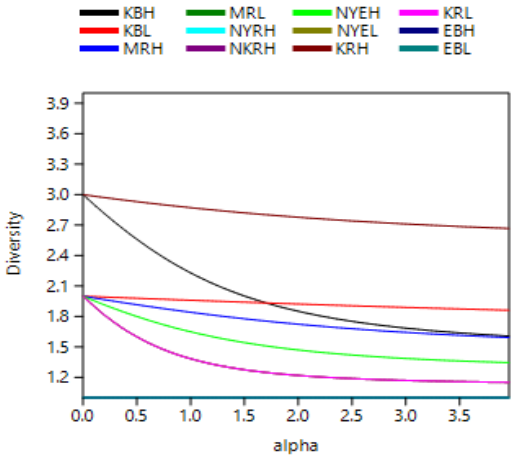

Fig. 5. Diversity profile of the *ralstonia* isolates based on sugar utilization

According to the Neighbor joining distance dendogram based on sugar utilization of the isolates, the various regions were grouped into four clusters (A, B, C, D). Cluster A included regions NYRH, EBH, MRL and EBL which were closely associated with isolates from regions KRL, NKRH and NYEL. Cluster B included regions KBH and MRH. Region C included KBL and KRH while Region NYEH was an independent out group and was placed in cluster D, (Fig. 6). Cluster C representing Kiambu lowland and Kirinyaga highland had significantly higher isolates of biovars 1 and 2 in comparison to all the other regions and had the highest diversity of the biovars isolated as shown in Fig. 6.

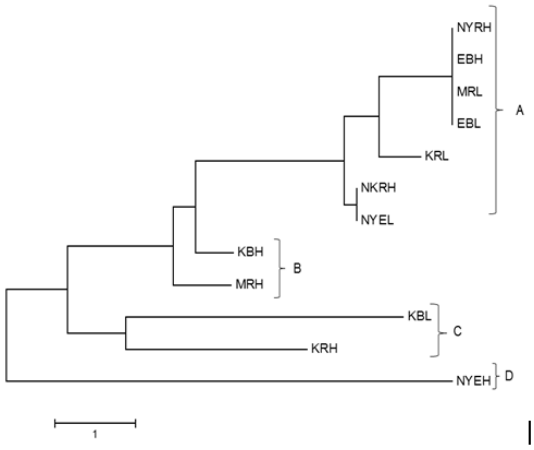

Fig. 6. Distance dendrogram drawn based on Neighbor joining method and Jaccard similarity index showing the diversity of the isolates based on sugar utilization

Kiambu and Kirinyaga regions are the most important potato and tomato producing regions in Kenya. Poor seed production systems common to these regions may contribute to the high diversity of *R. solanacerum* biovars observed. Reports of high transmission of the bacterium through infected seeds coming from the highlands are common [26,28,29].

R. solanacerum, has been found to express varied gene encoding cell wall and cell membrane synthesis, and similar to those that function in carbohydrate transport and metabolism. With this Milling's suggestion of presence of a great variation in chemical structure of the bacterium's extracellular polysaccharides, the two explain the high biochemical and pathogenic variability of this bacterium. The varied genes contribute to the complex difference in composition of extracellular polysaccharides, which contributed too to

disease development in the susceptible plants. In general, the identification of biovars affecting solanaceae crops in highlands and lowlands of Kenya is a step towards better techniques in management of the bacterial wilt disease in major solanaceae crops growing regions [24-26,28-29].

4. CONCLUSION

This survey findings indicated that *R. solanacerum*, biovars present in Kenyan Highlands and Lowlands included biovars 1, 2 and 3. These biovars are highly pathogenic on solanaceae crops throughout the world. The differential prevalence of this biovars, and their identity permits easy understanding of the pathogenicity of *R. solanacerum* for improved control measures of bacterial wilt disease.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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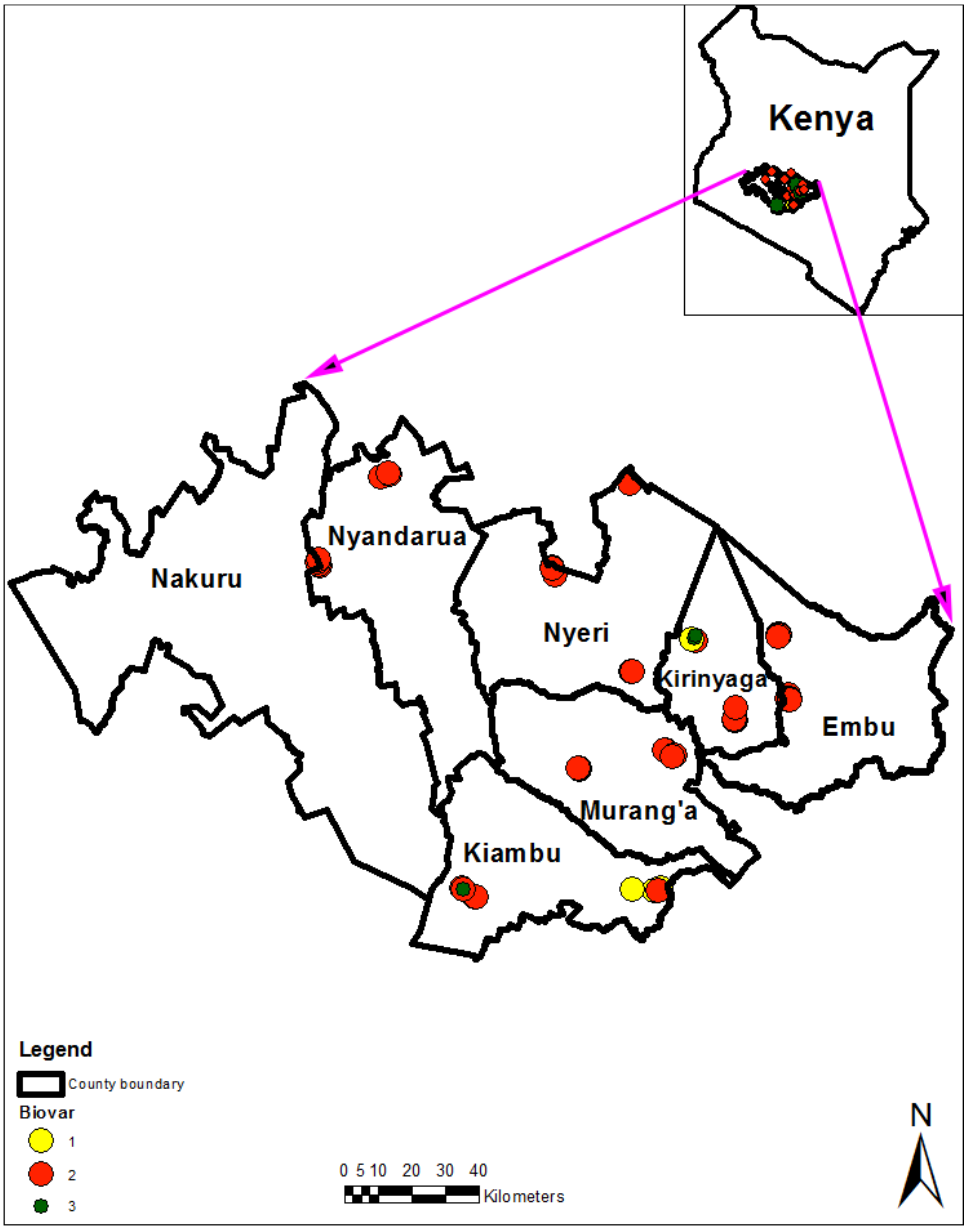
APPENDIX



Appendix 1. Map showing *R. solanacerum* biovars distribution in Kenyan highlands and lowlands

Appendix 2. Isolate diversity based on sugar utilization

Diversity indices	KBH	KBL	MRH	MRL	NYRH	NKRH	NYEH	NYEL	KRH	KRL	EBH	EBL
TAXA_S	3	2	2	1	1	2	2	2	3	2	1	1
INDIVIDUALS	10	10	10	10	10	10	10	10	10	10	10	10
DOMINANCE_D	0.54	0.52	0.58	1	1	0.82	0.68	0.82	0.36	0.82	1	1
SIMPSON_1-D	0.46	0.48	0.42	0	0	0.18	0.32	0.18	0.64	0.18	0	0
SHANNON_H	0.8018	0.673	0.6109	0	0	0.3251	0.5004	0.3251	1.055	0.3251	0	0
EVENNESS_E^H/S	0.7432	0.9801	0.921	1	1	0.6921	0.8247	0.6921	0.9572	0.6921	1	1
BRILLOUIN	0.5886	0.5347	0.4787	0	0	0.2303	0.3807	0.2303	0.8055	0.2303	0	0
MENHINICK	0.9487	0.6325	0.6325	0.3162	0.3162	0.6325	0.6325	0.6325	0.9487	0.6325	0.3162	0.3162
MARGALEF	0.8686	0.4343	0.4343	0	0	0.4343	0.4343	0.4343	0.8686	0.4343	0	0
EQUITABILITY_J	0.7298	0.971	0.8813			0.469	0.7219	0.469	0.9602	0.469		
FISHER_ALPHA	1.453	0.7517	0.7517	0.2766	0.2766	0.7517	0.7517	0.7517	1.453	0.7517	0.2766	0.2766
BERGER-PARKER	0.7	0.6	0.7	1	1	0.9	0.8	0.9	0.4	0.9	1	1
CHAO-1	3	2	2	1	1	2	2	2	3	2	1	1

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