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REGULAR ARTICLE

COMPARATIVE ANALYSIS ASSOCIATED TO VIRULENCE OF *PSEUDOMONAS SYRINGAE* PV. *GARCAE*, THE CAUSATIVE AGENT OF BACTERIAL BLIGHT OF COFFEE IN KENYA

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ABSTRACT

Given the immense damage and yield loss due to bacterial blight of coffee disease that is caused by *Pseudomonas syringae* pathovar *garcae*, this study sought to evaluate the diversity associated to the virulence of *Psg* isolates on coffee in Kenya. Twelve strains of *Psg* pathogen were collected from different coffee growing regions in Kenya and characterized using both phenotypic (host-pathogen interaction via laboratory inoculation) and molecular tools using and genomic sequencing. The sequencing was done using 16S ribosomal RNA primers 8 F and 1492 R and sequences were then retrieved for alignment and phylogenetic analysis using MEGA 6 via clustalW. The results revealed high variability of *Psg* isolates and possible existence of several races of *P. s. garcae* species. The study provides new knowledge on the nature of virulence of BBC pathogen, and a platform towards breeding for coffee with durable disease resistance in Kenya.

Keywords: Strains, Coffee cultivars, Virulence, Inoculation, Infection, Diversity

INTRODUCTION

Pseudomonas syringae is a gram-negative plant-pathogenic species comprising of at least 51 pathovars that can be distinguished by their range hosts and is best system for host specificity and virulence studies [1, 2]. The pathovars contain several races characterizable by their levels of virulence on different host cultivars [3] for a plant pathogen may be virulent causing disease on certain cultivars while avirulent on others.

Bacterial Blight of Coffee (BBC) caused by *Pseudomonas syringae* pathovar (pv.) *garcae* (here after *Psg*) is a major coffee disease [4] posing a great economic threat to coffee farming in Kenya especially in its hot spots regions. With the climatic changes experienced globally, the disease is increasingly becoming a major concern in Kenya in Arabica coffee [5] where it is responsible for crop losses mainly in windy and cooler cultivation areas. The pathogen directly infects succulent shoot tips during wet conditions at the temperatures of 15-25 °C coinciding with high relative humidity of about 80 % causing them to die-back. The incubation period under such field conditions is reported to be four weeks [6] with symptoms that include darkening, water-soaked necrotic lesions on leaves, tips and nodes of vegetative and cropping branches [4]. Infection of the shoots rather than leaves has been used as the major identification feature in Kenya and has been the basis of the disease assessment in epidemiology and chemical control studies [7]. The disease can cause total crop loss under conditions that favour the pathogen and the trees which are severely affected sometimes need to be destroyed [8]. Coffee growers have over the years relied greatly on copper-based formulations to control BBC which accounts for unsustainable (up to 30%) cost of production especially at the level of the small-scale farmers [9]. New disease patterns have emerged due to global climatic changes with observations of infection outside the regions where BBC was initially confined [6, 10]. Breeding for coffee varieties with durable resistance to the disease is desirable and is therefore a key mitigation measure towards addressing the challenge.

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The information on pathogenicity and virulence of this bacterium in Kenya is scanty since the pathogen has not been adequately studied. However, it has been demonstrated that Kenyan *Psg* isolates differed from the Brazilian isolates in terms of pathogenicity and biochemical characters [7]. The conclusion from that study alluded to the fact that the Kenyan isolates comprised of only one strain while the Brazilian comprised of one or more strains. The conclusion was drawn using only seven cultures used for that study from the National collection of Plant Pathogenic Bacteria, Harpenden, UK. This may not have been adequate representative for the Kenyan situation owing to the diverse climatic conditions within the coffee growing areas. The attributes of the global climatic changes may also have impacted greatly to pathogenic changes for adaptability and survival enhancement. It has not been established whether the occurrence of BBC in Kenya is caused by different strains of the *Psg* pathovar or not. It is therefore important to not only study the pathogenicity of this important pathogen, but also its diversity and interaction with Kenyan coffee in order to breed for durable resistance.

Ribosomal RNA is a universal tool for phylogenetic analysis, determination of interrelationships among organisms [11] and a good tool for identification and characterization of bacteria. The 16S rRNA gene sequences contain hyper variable regions that can provide species-specific signature sequences useful for bacterial identification [11] and capable of reclassifying bacteria into completely new species, or even genera and describe new species that have never been successfully cultured. Identification of DNA markers associated with virulence will be a great breakthrough. This study sought to isolate and characterize (through screening and sequencing), *Psg* isolates from diverse coffee growing regions with a view of establishing their genetic relatedness. Analysis on nature of interaction between the host plant versus the pathogen correlated with the molecular study providing knowledge on the nature of diversity of *Psg* pathogen.

MATERIALS AND METHODS

Collection of infected plant tissues and isolation of *Pseudomonas* spp

Plant tissues suspected to be infected with *Psg* (fig. 1) were collected from five different coffee growing zones, with the history of BBC, in Kenya. A summary of these areas, from which the samples were collected, is outlined in table 1. The infected twigs or shoots were cut using a sterilized pair of secateurs, placed in khaki paper bags and stored in an ice box. The samples were then taken to the laboratory for isolation and identification of the bacteria. To isolate bacteria, 7% Sucrose Nutrient Agar (SNA) medium was used as previously described [12].

Fig. 1: A coffee plant with symptoms of BBC infection which include dark, water-soaked necrotic lesions on leaves and branches culminating in a die-back

The lab code number was used to refer to the isolates

The isolated bacterial cells were allowed to ooze out into distilled water and films of the isolates streaked on the medium using a nichrome wire loop and incubated for 48 h at 25 °C. Single colonies of opaque ash-white, levan forming and mucoid pathogen were sub-cultured to obtain pure cultures.

Virulence test of the bacterial isolates

Four coffee genotypes namely SL 28, SL 34, Selection 6 and Batian 3 previously reported as susceptible to BBC [8] were used for virulence test. Coffee seedlings, approximately 4 mo old with 4 pairs of true leaves, were inoculated with a drop of approximately 5 µl of the bacterial suspension (10^9 CFU/ml) using the injection method [8] into the shoot primordial as shown in fig. 2.

The inoculated seedlings were maintained in a controlled growth room at temperature of 19-20 °C and relative humidity maintained at 75% [12]. It was expected that the isolates would exhibit similar characteristics on the same variety if they were one and same pathovars [7]. Symptoms of disease infection were recorded for three consecutive scores after every 7 d starting from the 14^{th} day after inoculation to determine progression of infection and severity of the disease.

Table 1: Details of suspected *P. s.* pv. *garcae* isolates collected from different coffee regions

No.	Source/county	Altitude a. s. l	Latitudes	Lab No.
1	Kapsabet/Kericho	1959m	0.10° N, 35.747° E	28/2012
2	Kisii	1700m	0.41° S, 34.47° E	37/2012
3	Nakuru	2001m	0.153° S, 36.138° E	44/2012
4	Mweiga/Nyeri	1939m	0.365° S, 36.91° E	51/2012
5	Kisii	1700m	0.41° S, 34.47° E	58/2014
6	Kapsabet/Kericho	1959m	0.10° N, 35.747° E	59/2014
7	Nakuru	2001m	0.153° S, 36.138° E	62/2014
8	Mweiga/Nyeri	1939m	0.365° S, 36.91° E	65/2014
9	Mweiga/Nyeri	1939m	0.365° S, 36.91° E	66/2014
10	Mweiga/Nyeri	1939m	0.365° S, 36.91° E	67/2014
11	Nyeri Hill/Nyeri	2200m	0.56° S, 36.93 ° E	68/2014
12	Nyeri Hill/Nyeri	2200m	0.56° S, 36.93 ° E	69/2014

A scale of class 1 to 5 was used classify the disease symptoms based on the degree of necrosis reached as earlier described [10] with modification, where: 1 = absence of the dark necrotic lesions, with yellow halo (bacterial blight); 2 = small black lesion; 3 = black lesion coalescing 4 =black coalesced lesion over 50% and 5 = complete girdling around the meristem. The data was subjected to analysis of variance (ANOVA). LSD5% was used to separate the means. The seedlings that scored ≤ class 2 were denoted as resistant; those that scored>2 but ≤3 as moderately resistants, while the ones that scored>3 denoted as susceptible.

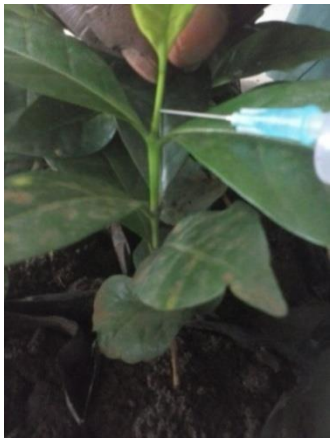

Fig. 2: Inoculation of a coffee seedling with BBC pathogen using the injection method

Fig. 3: Seedlings inoculated with BBC isolates showing symptoms of infection from class 1-5

Molecular characterization of different *Psg* isolates

DNA was extracted from bacterial isolates using the QIAprep Spin miniprep kit (Qiagen, USA) according to the manufacturer's instructions and the DNA confirmed by Agarose gel electrophoresis. To determine the diversity of the *Psg* cultures, amplification was done using 16S rRNA oligonucleotide primers; 8 F AGAGTTTGATCCTGGCTCAG and 1492 R (l) CGG TTA CCT TGT TAC GAC TT in a reaction mix containing Kapa Taq premix (Kapa Biosystems). The PCR amplification conditions were set at 94°C for 3 min followed by 30 cycles of 94°C for 30 seconds, annealing at 50°C for 30 seconds an extension of 72°C for 2 min and a final extension of 72°C for8 min. The PCR product was purified using a PCR purification kit (Qiagen, USA). The DNA samples were sequenced using the same 16S primers. Sequences were retrieved and compared automatically using the BLAST against the sequences of bacteria available in databanks (http://www. ncbi. nlm. nih. gov/) to obtain related sequences. They were then used for multiple sequence alignment using vector NTI software version 11.0 (Invitrogen, USA). The alignments were then used to determine the genetic evolutionary relatedness using clustal W [13] in Molecular Evolutionary Genetic Analysis 6 (MEGA 6). The Neighbor-Joining algorithm was used to determine pairwise distances and the evolutionary history inferred using the Maximum Likelihood method based on Tamura and Nei model [14]. A phylogenetic tree was constructed using Boostrap method with 500 replications with gap opening penalty of 15 and gap extension penalty of 6.66. All positions containing gaps and missing data were eliminated [15].

RESULTS

Psg was identified as a Gram-negative, rod-shaped, obligate aerobe and flagellated motile bacteria. Disease symptoms started to be visible after 5 d from inoculation with signs of wilting and water soaking (fig. 3) with black lesions observed at the progressed disease stage. Isolates from Kisii-58/014, Mweiga-66/014, Nakuru-62/014 and Nakuru-44/014 in that respective order were the most virulent. Isolates from Nyeri-69/014, Kapsabet-28/012 and Kisii-37/012 caused the least infection which closely compared to the control. The analysis revealed significant difference ($P<0.05$) among the 12 isolates used (table 2) depicting evident variability. It was observed that some isolates from the same region expressed different virulence levels as demonstrated by isolates Kisii-58/014 (4.60^{a}) and Kisii-37/012 (1.85^{efg}) both from the same locality. The same case was exhibited by the five isolates from Nyeri region where isolate Nyeri-69/014 (1.57^{fg}) very mild compared with isolate Nyeri-66/014 (4.13^{ab}) from the same region which was among the most virulent. The two isolates from Kapsabet [Kap-59/014 (3.48^{bc}) and Kap-28/012 (1.82^{efg})] were significantly different in their virulence. However, the isolates Nakuru-62/014 (4.08^{ab}) and Nakuru-44/012 (3.82^{ab}) had no significant difference in their virulence. The results therefore revealed both localized and regionalized difference of the isolates in terms of their virulence opening discussion on the course and extent of the diversity.

Table 2: Virulence means of the BBC isolates against selected coffee genotypes

No.	Isolate	Virulence
1	Kisii-58/014	4.60 [a]
2	Mweiga-66/014	4.13 [ab]
3	Nakuru-62/014	4.08 [ab]
4	Nakuru-44/012	3.82 [ab]
5	Mweiga-67/014	3.62 [bc]
6	Kap-59/014	3.48 [bc]
7	Nyeri-68/014	2.95 [cd]
8	Mweiga-65/014	2.43 [de]
9	Mweiga-51/012	2.33 [def]
10	Kisii-37/012	1.85 [efg]
11	Kap-28/012	1.82 [efg]
12	Nyeri-69/014	1.57 [fg]
13	Control	1.48 [g]
	LSD(0.05)	0.84
	CV (%)	35.23

*Means followed by the same letter indicate no significance difference according to Duncan's test

The isolates were categorised in 4 major groups representing the level of their virulence fig. 4. The number of seedlings inoculated per isolate (15seedlings per replication x 4 coffee genotypes) were categorised into resistant seedlings (classes 1 and 2) and susceptible seedlings (classes 3, 4 and 5). The isolates from Nyeri-68/014, Nakuru-62/014, Kisii-58/014, Mweiga-66/014, Mweiga-67/014, Kap-59/014 and Nakuru-44/012 had over 50 seedlings (83%) scoring between classes 3-5 of the disease symptoms (fig. 5).

Isolates Kap-28/012, Kisii-37/012, Mweiga-51/012, Mweiga-65/014 and Nyeri-69/014 had least infection with an average of 97% of the total seedlings inoculated scoring class 1 and 2. The interaction of the genotypes and the isolates had no significant difference. Batian 3 scored the lowest disease mean of 2.72 followed closely by Selection 6 with a mean of 2.81. The other traditionally grown Kenyan commercial varieties SL 28 and SL 34 had a mean of 3.06 and 3.15 respectively. The two later varieties are known to be susceptible to the other major coffee diseases in Kenya namely Coffee Berry Disease (CBD) and Coffee Leaf Rust (CLR) both fungal diseases. The performance of the 12 BBC isolates appeared to be similar across the four coffee varieties (table 3).

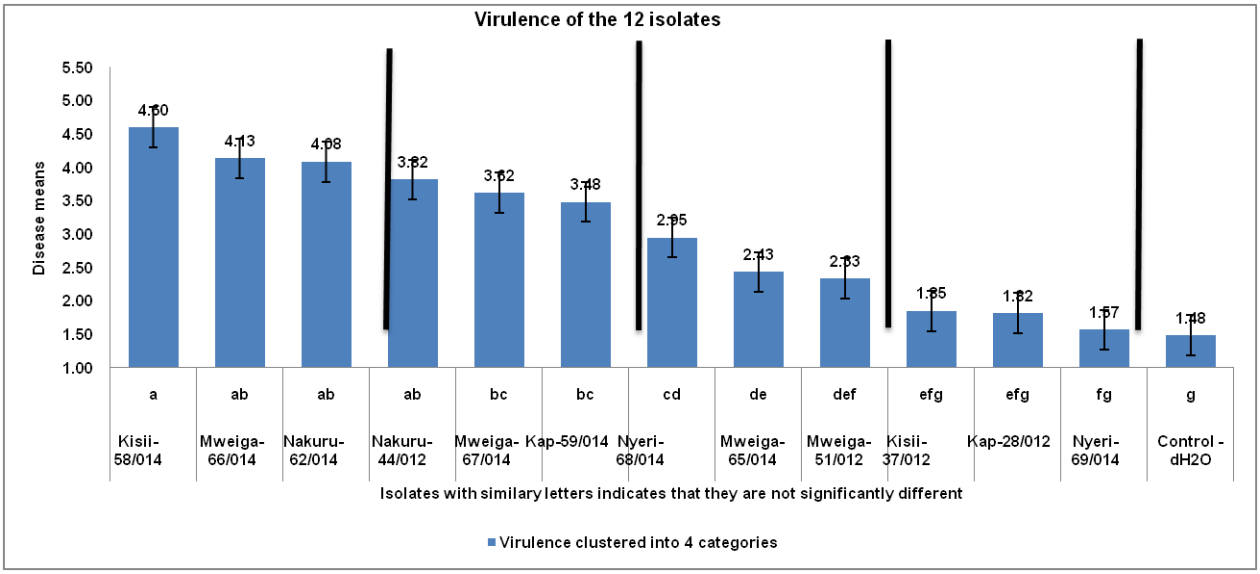

Fig. 4: Virulence means of the 12 BBC isolates

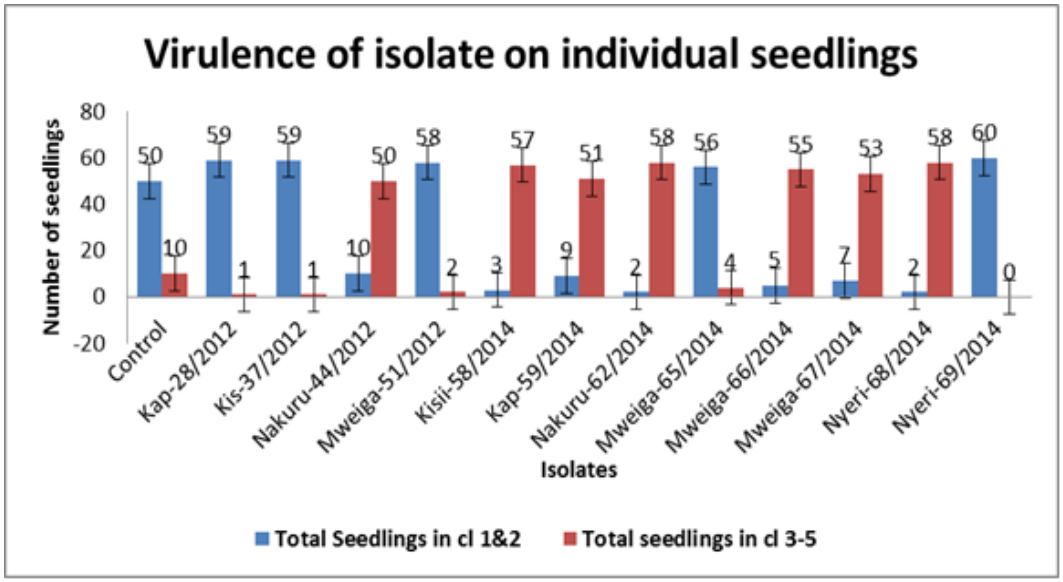

Fig. 5: Distribution of resistant and susceptible seedlings inoculated with the 12 BBC isolates-Seedlings in class 1 and 2 were resistant while those in classes 3, 4 and 5 were susceptible. The more seedlings that scored class 3 to class 5, the more virulent an isolate was considered to be

Table 3: Reaction of the 4 genotypes used to determine virulence of the 12 isolates

Rank	Genotype	Mean
1	SL34	3.15 [a]
2	SL28	3.06 [a]
3	Sel 6	2.81 [a]
4	Batian 3	2.72 [a]

CV=35.232781% LSD 0.05 = 0.46462403115, *Means followed by similar letter indicate that there is no significant difference in Duncan's test

Half of the seedlings inoculated with the isolates were classified as susceptible. Isolates Kap-28/012, Mweiga-65/014 and Nyeri-69/014 caused minimum disease infection on Batian 3. However, Isolates 58/014 and 62/014 and 67/014 were the most virulent to Batian 3 with all seedlings scored falling between classes 3 to 5. Isolates Nyeri-69/014 had no seedlings scored in class 1 or 2 in all the four varieties showing the high level of virulence.

Characterization of the different *P. s.* pv. *garcae* isolates using 16S gene

All the isolates were amplified with the 16S rRNA primer (fig. 7) and the DNA bands were estimated to be 1200 base pairs and therefore adequate for sequencing. There were a total of 1056 positions in the final dataset using the forward primer and 1066 using the reverse primer. This confirmed that the estimation of the bands weight after amplification was accurate.

The analysed data generated from the sequences revealed high diversity in evolutionary relationship among the 12 *Psg* isolates. There was close similarity in the way the sequences from the 12 isolates were phylogenetically clustered and their virulence levels as shown in fig. 8. The most virulent isolates which had caused highest disease infection of more than 3.48 were clustered together in one group.

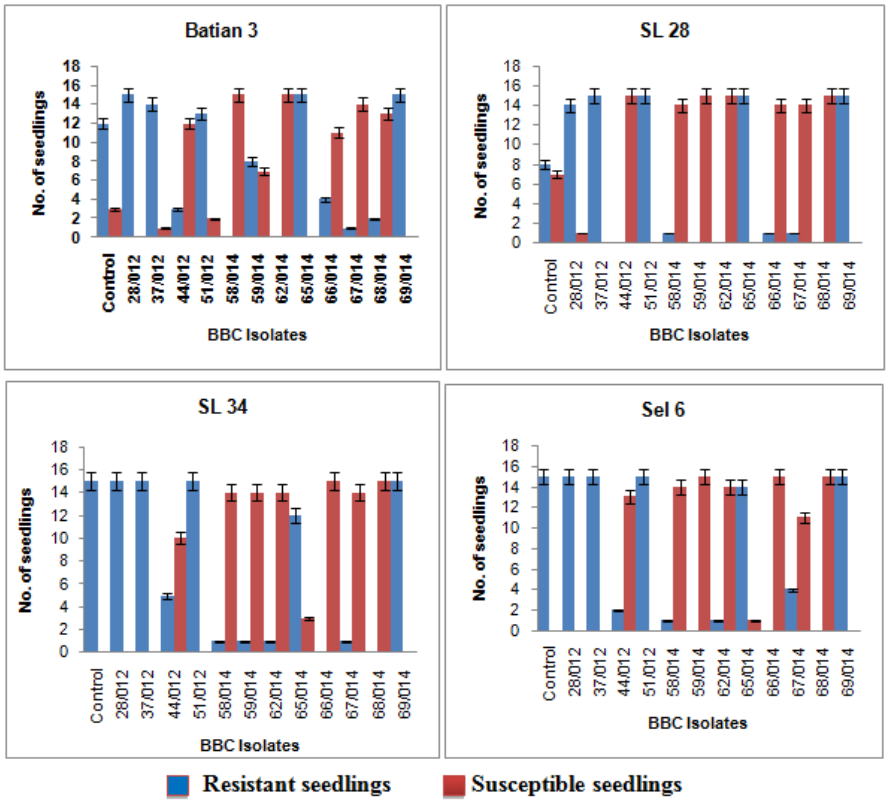

Fig. 6: Distribution of resistant and susceptible seedlings of the 4 test genotypes

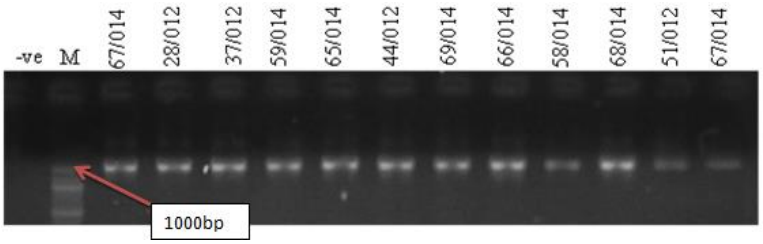

Fig. 7: DNA Amplification of the 12 BBC isolates with 16S rRNA primer

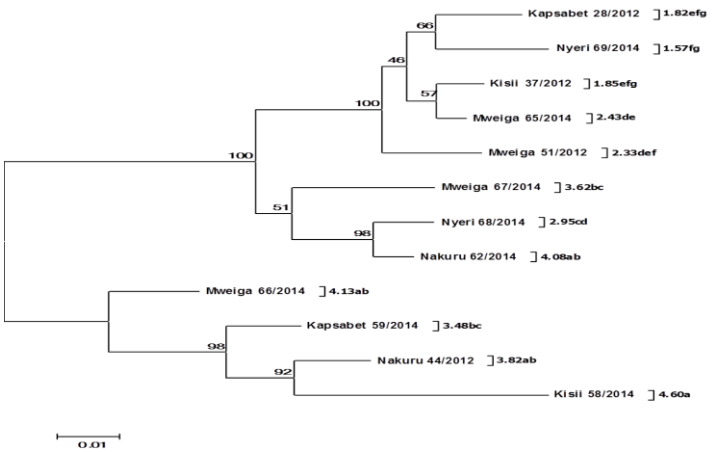

Fig. 8: Molecular phylogenetic analysis by maximum likelihood of nucleotides sequence of 16S rRNA gene. The fig. **following the label of the isolates represents the virulence mean of the isolates with the significant number according to Duncan's test. The tree with the highest log likelihood (-3648.2399) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown above the branches [15]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved the 12 isolates of *Psg* nucleotide sequences**

These isolates include isolate Kisii-58/014 with a disease mean of 4.60[a], Nakuru-44/012 (3.82[ab]), Mweiga 66/014 (4.13[ab]) and Kapsabet-59/014 (3.48[bc]). Two isolates with high disease virulence namely Nakuru 62/2014 (4.08[ab]) and Mweiga 67/2014 (3.62[bc]) were however clustered in a different group but closely linked to the most virulent group. Both forward and reverse primers also clustered isolate Kapsabet 28/2012 (1.82[efg]), Nyeri 69/2014 (1.57[fg]) and Kisii 37/2012 (1.85[efg]) which were the least virulent isolates together. The four isolates from Mweiga were also clustered according to their virulence with isolate Mweiga-66/014 which had caused the highest disease infection score of 4.13[ab] from that region separated from the others. The two Isolates from Kisii [Kisii-37/012(1.85[efg]) and Kisii 58/014(4.60[a])] were quite diverse just as in their intensity to cause disease. The same was replicated in Kapsabet isolates (Kap-28/012 and Kap-59/014) where there appeared to be no link between them. Out of the 1263 nucleotides sequenced that translate to 421 proteins and aligned, 357 were conserved sites, while 894 were the variable sites and 835 Parsimony-informative sites. Distinct variability between the most virulent and the less virulent isolates was observed at the codon site 247, 248 and 249 nucleotides.

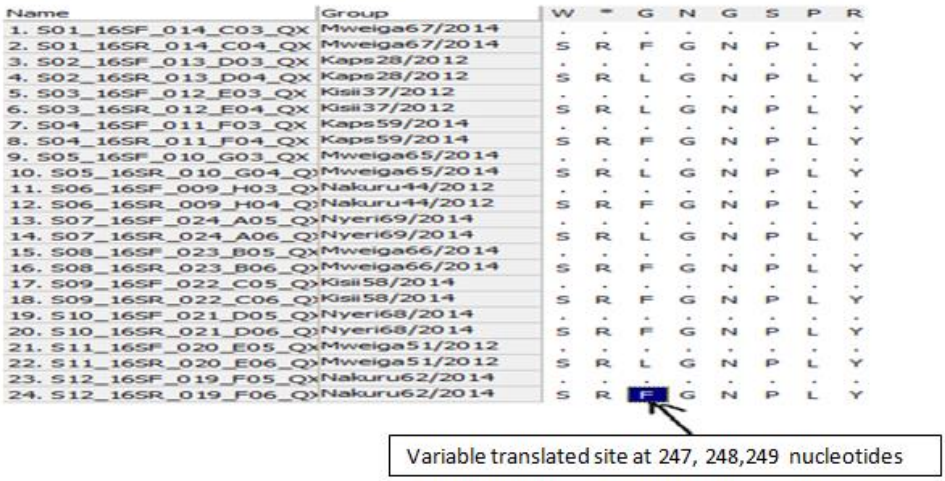

Fig. 9: A region of the aligned translated sequence with high variability

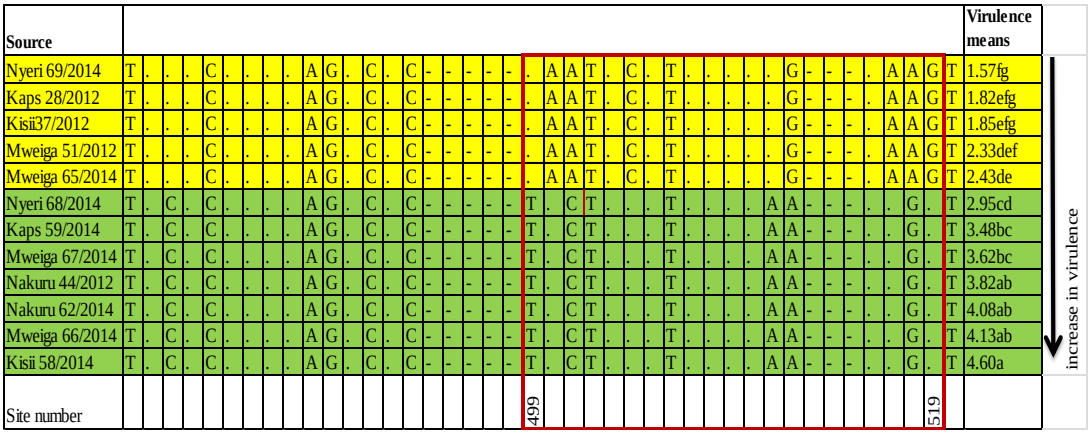

Fig. 10: A region of aligned DNA sequences of the 12 *Psg* isolates showing variable sites closely linked to disease virulence mean between 499 and 519 nucleotides

The isolates which had a disease virulence means of 2.95 and above are designated with letter F while the ones with a lower virulence are designated with letter L at site 247, 248 and 249 nucleotides in fig. 9. A similar nucleotide pattern was observed at the site between 499 and 519 (fig. 10). All the sequences of the most virulent isolates with more than 2.95 disease means had T. CT...T.... AA.... G. at the site between 499 to 519 nucleotides presenting a high variability from the least virulent isolates which had. AAT. C. T.... G.... AAG.

DISCUSSION

The disease symptoms started to be observed on day five (5) from inoculation as had earlier been reported [8] and all the isolates were found to be pathogenic. The difference observed amongst the isolates (table 2) could largely be contributed to virulence factors in the isolates used in the study. It has been reported that the outcome of host-pathogen interactions is controlled by compatibility factors, which consist of virulence factors in the pathogen and susceptibility factors of the host [16]. The difference of isolates collected from the same region was also evidently

observed with the example of isolates from Kisii, Kapsabet and Nyeri which had significant difference. There appears to be several avirulence genes controlling virulence of the *Psg* which may be due to their distinguished races [17].

Ribosomal RNA has proved to be a universal tool for the phylogenetic analysis and interrelationship among organisms. About 1,500 nucleotides are present in 16s r-RNA which are determined using polymerase chain reaction (PCR) and gene sequencer [18]. The 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification [19]. There was high similarity between the phylogenetic analysis and the host pathogen interaction as isolates were clustered to a great extent according to their virulence. The 16S sequencing is capable of reclassifying bacteria into completely new species, or even genera [20]. It has also been used to describe new species that have never been successfully cultured. The results revealed the existance of great diversity among the *Psg* found in Kenya as had earlier been reported [12]. The difference of isolates collected from the same region was also observed with the example of isolates from Kisii, Kabsabet and Nyeri which had significant difference. This could also imply the existance of different strains of *Psg* in Kenya. Evolutional impact within the garcae species may have yeilded different strains and their increasing the risk of Kenyan coffee to BBC infection. The commercial varieties lay exposed due to their inability to resist the bacterial infection. Although great diversity among the *Psg* isolates was evident, however, there was high sequence homology among all the isolates evaluated at 61% at 100% level as expected since they belong to the same pathover.

These results indicate the high virulence and variability in the Kenyan *Psg* populations, and reveal vulnerabilities of elite germplasm to the possible diverse strains. Breeding programme to introgres resistance gene into the commercial varieties through pyramiding of resistance genes could offer durable resistance to the coffee. Identification and selection of coffee genotypes with horizontal resistance to BBC strains for inclusion in the breeding programme is therefore important. A nomenclature of the possible different strains of *Psg* in Kenya need to be developed and a continous pathogen monitoring and more possible strains identification be embraced.

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AUTHORS' CONTRIBUTIONS

Ithiru J. Mwangi, Gichuru E. K-Virulence test of the bacterial isolates

Ithiru J. Mwangi, Masanga J, Alakonya A. E-Molecular characterization of the pathogen

Ithiru J. Mwangi, Gichuru E. K, Alakonya A. E-Data analysis, discussion and manuscript writing.

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