Vol. 23(5), pp.188-201, May 2024
DOI: 10.5897/AJB2023.17588
Article Number: C98296672184
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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African Journal of Biotechnology

Full Length Research Paper

Pathogenic and molecular characterization of *Pseudocercospora griseola*, the causal agent of angular leaf spot of common bean, in Eastern Democratic Republic of Congo (DRC)

Kijana Ruhebuza[1]*, Mathew Abang[2], Richard Edema[3], Clare Mukankusi[2], Fred Kato[2] and Robin Buruchara[4]

[1]Institut National Pour l'Etude et la Recherche Agronomiques (INERA) MULUNGU, D/S Bukavu, Democratic Republic of the Congo.
[2]Centro International for Tropical Agriculturale (CIAT) NARL Kawanda, Kampala P. O. Box 6247, Uganda.
[3]Makerere University, Kampala P. O. Box 7062, Uganda.
[4]Centro International for Tropical Agriculturale (CIAT), Africa Regional Hub, Nairobi, Kenya.

Received 20 June 2023; Accepted 30 August 2023

Angular leaf spot (ALS) caused by *Pseudocercospora griseola* is the most economically damaging common bean (*Phaseolus vulgaris* L.) foliar disease in Democratic Republic of Congo (DRC) and elsewhere in Eastern and Central Africa. Information regarding pathotypes distribution and genetic structure that would enable development of appropriate control measures is still lacking in DRC. The study aimed to investigate the pathogenic and genetic structure of *P. griseola* isolates from two agro-ecological zones of eastern DRC. Virulence analysis of 59 *P. griseola* isolates, using differential cultivars, identified 35 pathotypes groups. Most virulent pathotypes were discerned in sub-humid highlands area: Repetitive PCR primers, BOXAIR and REP used to elucidate genetic diversity revealed high level of polymorphism (95.5%), influence of agro-ecology and host genotypes on *P. griseola* population structure; with moderate genetic differentiation, respectively (G_{ST}= 19% and $G_{ST=}$ 12%). Low genetic diversity(D_{ST}) value revealed that most of the genetic variability was within subpopulations (96%), with little variability between *P. griseola* sub populations from the host genotypes (4%). The cluster analysis using UPGMA method identified two main clusters at 65% genetic similarities. The study confirmed that *P. griseola* populations from eastern DRC, can be broadly grouped into two structured host genotype groups and co-evolution process. This implies that screening for ALS resistance should consider this high diversity for an appropriate breeding program and durable disease management strategies development.

Key words: Pathotypes, agro-ecology, common bean, genetic distance, durable resistance.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L) is the most important food legume crop in the great lakes region of

*Corresponding author. E-mail: kijanaruhebuza@gmail.com. Tel: +243852010252.

central Africa (Blair et al., 2010*)*, where it is the second human source of dietary protein and the third most important source of protein, carbohydrates (Gudero and Terefe, 2018) and vital micronutrients (Nay et al., 2019) for lower incomes strata (Schwartz and Pastor Corrales, 1989). The highest per capita consumption ranging from 31 to 66 kg per year has been reported in Central and Eastern Africa countries (Petry et al., 2015). In Democratic Republic of Congo (DRC), mainly in Eastern provinces, North and South Kivu, consumption is estimated at 300 g/capita per days (http://www.harvestplus.org/content/iron-beans-dr-congo). Democratic Republic of Congo is among the largest common bean producers in Eastern and Central African (Beebe et al., 2014; Petry et al., 2015), with common bean production estimated to 248.957 metric tons, and 459.100 ha of land under bean production (FAOSTAT, 2014). The crop is grown for cash as well as for food security USAID (2010). It is grown by small scale farmers with low inputs, in diverse environmental conditions and cropping system (Wortmann et al., 1998). Eastern part of the DRC is a main production area contributing to more than a half of the total country bean production (USAID, 2010).

However, common bean production is hampered by several abiotic and biotic constraints. As food demand continues, agricultural production is further threatened (Santos and Silva, 2023). Angular leaf spot (ALS) disease caused by *Pseudocercospora griseola* Sacc. (Crous et al., 2006) is among the major biotic stresses (Ngeleka, 2006; Lemessa et al., 2011; Getachew and Habtamu, 2018), that cause major yield losses in common bean crop (Almeida et al., 2022). Yield losses estimated to be 374 800 tons/year was reported in Eastern and Central Africa (Wortmann et al., 1998), furthermore a significant impact of angular leaf spot disease on common bean productivity in Africa, causing up to 80% yield losses was mentioned by Ddamulira (2019). It is also a serious bean constraint in tropical and subtropical areas (Nay et al., 2019). It was reported in more than 60 countries worldwide (Guzmán et al., 1995). A 90% angular leaf spot incidence was reported in farmers' fields in the Eastern Democratic Republic of Congo, North and South Kivu provinces (Kijana et al., 2017). Angular leaf spot causes symptoms on leaves, petioles, pods and branchs (Lemessa et al., 2011). Lesions on pods appear oval to circular with reddish brown centers. Infected pods may contain poorly developed or shrivelled seeds and may carry the fungus (Stenglein et al., 2003). An array of conventional and molecular techniques, including race analysis with a known set of host differentials and molecular tools has been used to generate information regarding the *P. griseola* pathogen variability (Mahuku et al., 2002a). Molecular data are important to accurately identify the species and also identify species bounderies and potential risk (Bhunjun et al., 2021). It is important to understand genetic variation in the nature; moreover, molecular markers are becoming increasingly relevant for studying taxonomic and evolutionary relationships among various fungi (Seethapathy et al., 2022). Despite the availability of molecular tools, phenotypic evaluations under field conditions will remain an important method and the final quality check for selecting new varieties (Nay, 2019). Such information is helpful for understanding pathogen population structure, and how virulence is related to profiles revealed by molecular markers, in order to develop sustainable control measures (Bhunjun et al., 2021).

Variability of this fungus was demonstrated using isoenzymes markers (Boshoff et al., 1996); these markers allowed separation of 55 *P. griseola* isolates into two groups; whereby 26 isolates from Africa clustered in one group, while USA and Latin American isolates were distributed across the two groups Correa-Victoria cited by Crous et al. (2006). Various others molecular markers have been used to elucidate genetic variability in *P. griseola*, such as Random Amplified Polymorphism DNA (Nietsche et al., 2001), Inter-Simple Sequence Repeats (ISSR) (Stenglein and Balatti, 2006; Abadio et al., 2012), and Restriction Fragments Length Polymorphism of ribosomal intergenic spacers region (IGS-RFLP) (Mahuku et al., 2002a). The studies confirmed the high diversity of *Pseudocercospora griseola* pathogen populations. Improved understanding of the co-evolution of beans and *P. griseola* led to the development of a set of 12 differentials cultivars (6 Andeans and 6 Mesamericans) proposed by Pastor-Corrales and Jara for characterization of *P. griseola* pathotypes (BIC, 2011). This method confirmed specialization of the pathogen to their respective common bean host gene pools and the co-evolution process (Pastor Corrales et al., 1998; Nietsche et al., 2001) as well as distribution of pathotypes according to agro-ecological zone, at different spatial scales (Guzmán et al., 1995; Aggarawal et al., 2004; Pastor-Corrales, 2004; Sartorato and Alzate-Marin, 2004). Using this set of differentials cultivars, a number of studies including Sartorato and Alzate-Marin (2004) and Orozco and Araya (2005) demonstrated the highly variability of this pathogen. A study by Mahuku et al. (2002a) revealed that a group of Andean isolates was able to infect Mesoamerican differentials cultivars, among the African isolates using a set of 12 differentials cultivars, Random Amplified Microsatellites (RAMS) and (IGS-RFLP) analysis. This group of isolates was designated as Afro-andean. Among the African isolates, four isolates from DRC that were classified either Andean or Mesoamerican, none belonged to the Afro-andean sub-group of isolates. Earlier, Pyndji (1992) had identified 17 pathotypes, among the 21 isolates from the Great Lakes Region (Rwanda, Burundi, Democratic Republic of Congo), using 11 differentials cultivars. Apart from identification of pathogenic groups/races among a very limited sample of isolates collected from DRC, little

information was still known about the population and pathotypes structure of *P. griseola* in the country. Population structure refers to the amount of genetic variation among individuals in a population, partition of variation in space, and the phylogenetic relationships between individuals within and between pathogen sub-populations (Leung et al., 1993). While, pathotypes structure refers to race composition of the pathogen and the geographic distribution of different physiological races (Bennett, 1993). Because of the high virulence diversity of *P. griseola* and the great potential to overcoming resistance (Nay et al., 2019), the most effective strategy for controlling this disease is the use of genetic resistance, the least expensive, safest and the most practical for common bean producers (Taboada et al., 2022; Pádua et al., 2022; Santos and Silva 2023). Successful breeding and deployment of durable host resistant depends on an understanding of variation in pathogen population, virulence diversity as well as evolution of this pathogen (Abadio et al., 2012; Nay et al., 2019). This is a prerequisite for sustainable disease management strategies development.

The objective of this study was to investigate the pathogenic and genetic variability of *P. griseola* in eastern DRC, to contribute to the development of improved disease management strategies and ultimately to increase bean productivity in eastern DRC.

MATERIALS AND METHODS

Pathogen isolation and inoculums preparation

A pathogen collection survey was conducted in North and South Kivu regions that represent two bean environments of eastern DRC, in sub-humid highlands in low latitude (South Kivu) and sub-humid middle-altitude in low latitude (Nord Kivu) (Figure 1) by a team of bean researchers. Farmers' fields were visited randomly and leaf samples were collected from naturally infected bean plants that exhibited well developed symptoms. We collected leaves that were not in touch with the ground to avoid carrying out of other pathogens. Samples were collected from both Andean genotypes (large seeded) and Mesoamerican host genotypes (small seeded). Plant samples were kept in well aerated clean dry paper bags and transported to Uganda, where pathogen isolation, inoculum production and disease evaluation were conducted at Center International for Tropical Agriculture (CIAT) station that is based at the National Agricultural Research Laboratories (NARL). The following protocols defined by Pastor-Corrales et al. (1998), with minor modifications as described here were used. Synemmata were picked from the underside of the leaves using a needle under a compound microscope and placed on V8 juice-agar media amended with rifamycin and cultures allowed to grow for 1 to 3 days. Thereafter, single spores were transferred to V8 media without any antibiotics and monosporic cultures grown. Inoculum was produced from each monosporic *P. griseola* isolate growing in a Petri dish by adding sterile distilled water and scraping the surface of the plate to dislodge the conidia. The conidial concentration was determined using hemacytometer, then adjusted to 2×10^4 conidia/ml (Pastor-Corrales et al., 1998). *P. griseola* isolates collected were given an ID/code.

Determining virulence phenotypes and races of *P. griseola*

For virulence analysis, each monosporic isolate was inoculated onto a set of 12 common bean differential genotypes in the greenhouse. The differential set includes six large seeded of Andean origin. The genotypes are the following Don Timoteo, G117963, Bolón Bayo, Montcalm, Amendoin and G 5686 and six small or medium sized as Middle American genotypes such as: PAN 72, G 2858, Flor de Mayo, Mexico 54, BAT 332 and Cornell 49242. Five grains of each differential cultivar were planted in a 5 L plastic bucket/pot in a screenhouse. The experiment was laid out in split plot design with 3 replications, isolates being main plot and genotypes/differentials cultivars sub-plot. Each replication was represented by one pot of 5 plants. Inoculation and disease evaluation was done according to Pastor Corrales et al. (1998) method and described here. Seventeen days after planting, when most cultivars had one fully expanded trifoliate leaf, conidial suspension adjusted to 2.4×10^4 conidia/ml were sprayed onto the upper and lower surfaces of leaves of the host differentials. Inoculated plants were covered with polyethylene bags, to maintain high relative humidity ≥80-90%. Care was taken to avoid cross contamination between the isolates by disinfecting the spray bottles after each inoculum application. Polyethylene bags were removed after 4 days, then inoculated plants were exposed in the greenhouse at 24 to 30°C for about 10 days, until symptoms were fully expressed. Disease evaluation on individual plants were made at 10, 13, 17, 19 and 21 days after inoculation, using 1 to 9 CIAT rating scale, where 1=immunity no visible symptoms: no visible lesions, 3 resistant represent few and generally small lesions on most leaves, covering 2% of the foliar area, 5 intermediates represent lesions covering about 5% of the foliar area, 7 susceptible: presence of mostly large lesions surrounded by chlorotic halos, covering approximately 10% of the foliar area, and 9 highly susceptible, presence of very large lesions surrounded by chlorotic halos, covering 25% or more of the foliar area, sometimes with defoliation (Schoonhoven and Pastor-Corrales, 1987). Scores recorded on days 21 was often the highest; these were used in this study. Host responses were judged as susceptible, when disease score was 7 or higher during any evaluation, all other responses were judged as resistant (scores of 1 to 3) or intermediate (scores of 4 to 6).

Data analysis

Data obtained from disease severity evaluation on 21th days, when a variety was assigned a score > 3, then binary race analysis was performed, that consisted of assigning a binary number to each variety of the six belonging to Andean group and the same for the six varieties belonging to Mesoamerican group; as the following: Variety 1, number one was called a=Don Timeteo, was assigned the binary value 1, a=1, b=2, c=4, d=8, e=16, f=32. The sum 1+2+4+8+16+32= 64. The sum of binary values assigned to each group was used to test virulence level of each isolate and to cluster isolates into pathotypes. For example, we used two numbers separated by a dash, race 61-55 for isolate SK 210. The first number 61 was obtained by adding the binary values of the susceptible Andean differential cultivars, each of which was given a letter: acdef: 1+4+8+16+32=61. The second number 55 was obtained by adding the binary values of the susceptible Middle American cultivars: ghikl: 1+2+4+16+32=55 (Pastor-Corrales et al., 1998). Isolates with similar binary values for the two set of differential cultivars (Andean or Mesoamerican) were ranked into the same pathotype group. Care was taken to mention on which genotype group, Andean or Mesoamerican isolates were collected. During evaluation and data analysis, when an isolate affected all or more large seeded genotype, it was designed as Andean, when it

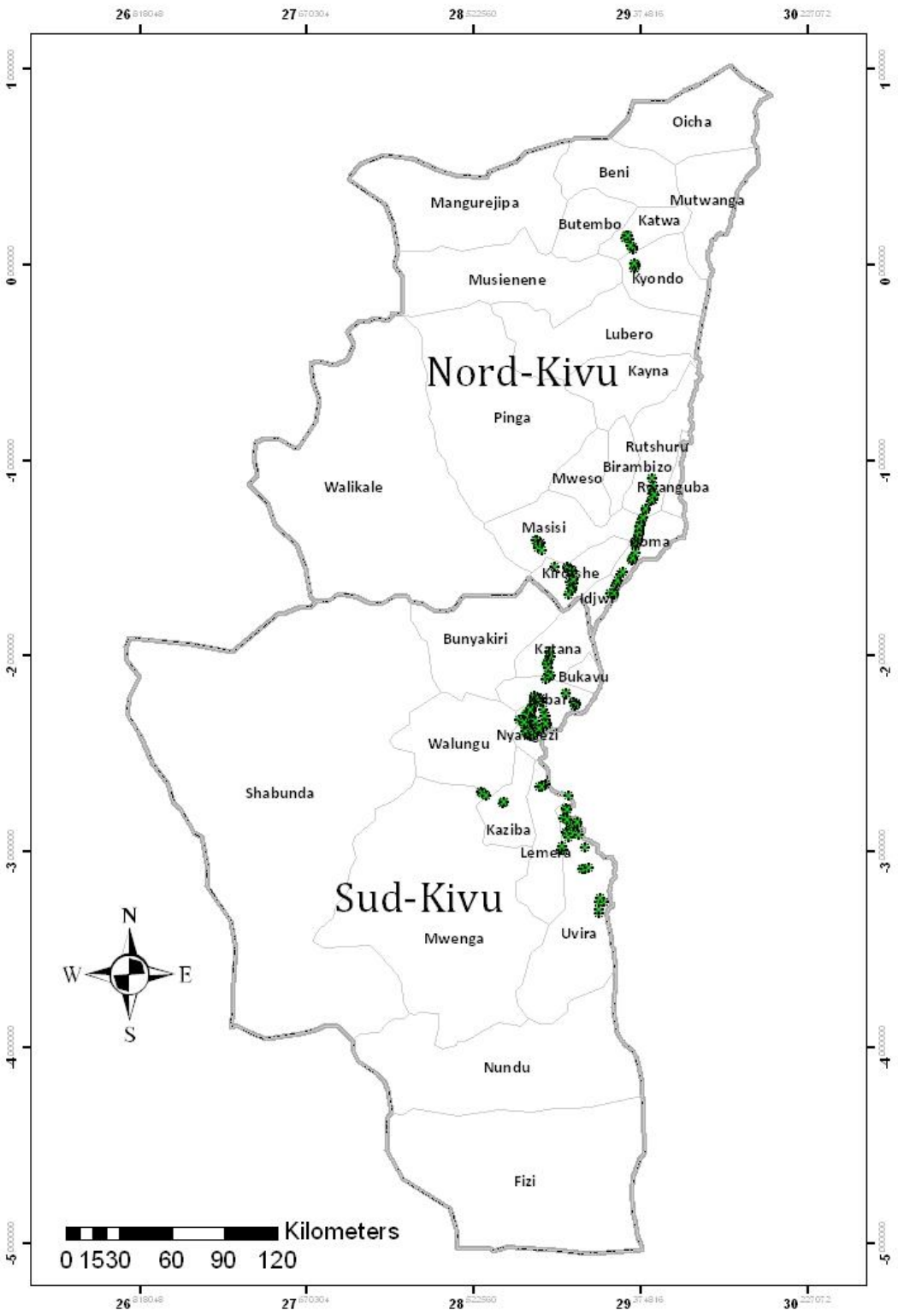

Figure 1. Map showing sites where plants affected by ALS disease were sampled in Eastern DRC.

affected all or more small/medium seeded/genotypes, it was designed as Mesoamerican. However, Afro-Andean isolates were collected on Andean genotypes, but affected both Andean and Mesoamerican differential cultivars.

Furthermore, analysis of the virulence data was done using ANOVA from Genstat software 13.0. The rate of infection was

calculated, taking into account the different observations time. They were summarized in relative area under disease progress curve (Simko and Piepho, 2012), using the following formula: rAUDPC = AUDPC / (Total length of evaluation × Maximum score on the scale), where AUDPC= [(Di+Di-1) × (ti-ti-1)] / 2 (Mahuku et al., 2002b). These rAUDPC were then used for analysis of variance by Genstat software version 13.0.

Molecular characterization

DNA extraction

DNA (Deoxyribo Nucleic Acid) was extracted using Sodium Dedocyl Sulfate (SDS) method described by Mahuku *(*2004). DNA was eluted from the pellet using 100 µl of TES ([0.2 M Tris HCl, PH 8), 10 mM EDTA (PH 8), 0.5 NaCl 1% SDS) or 60 µl of Polymerase Chain Reaction (PCR) water. DNA concentration was quantified using spectrophotometer, a nanodrop (Thermo scientific, Walthan, MD, USA). The resulting concentration was standardized to 20.0 ng/µl. To elucidate genetic diversity of *P. griseola* from different agro ecological zones of DRC, where common bean is an important crop and Angular leaf spot as a major disease and to determine how variability within *P. griseola* is structured. Genomic fingerprints were generated via polymerase chain reaction (PCR) using primers derived from the DNA sequence of repetitive elements (Rep-PCR), for Rep primers derived from repetitive extragenic palindromic (REP) sequence and conserved repeated bacterial DNA elements "BOX" primers derived from the enterobacterial repetitive intergenic consensus (ERIC) (Versalovic et al., 1994). Rep-PCR conditions: REP, ERIC AND BOXA1R-PCR were performed in 25 µl final volume containing 1x PCR buffer with $MgCl_2$, 1.25 mM dNTPs, 0.20 U of Taq polymerase, 1 µM of either one primer (BOXA1R, REP or ERIC), 10x dimethyl sulfoxide (DMSO), 0.16 mg/ml of bovine serum albumin (BSA), 80 ng genomic DNA sample and PCR water (Versalovic et al., 1994). Amplification was done in a thermocycler (Bioneer mycycler, Korea) programmed for initial step of DNA denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 42°C for REP primers, and 50°C for ERIC, BOX primer for 30 s. The extension was done at 65°C for 5 min, with a final extension at 65°C for 16 min, then hold at 4°C, until electrophoresis. Table 2 shows sequences and annealing temperature of repetitive primers used.

Electrophoresis and visualization

Deoxyribo Nucleic Acid (DNA) fingerprinting products from repetitive primers were separated on 1.6% agarose gel at a constant 100 V, in 1 × Tris Borate Ethidium (TB buffer), and stained with ethidium bromide, then visualized using Genbox gel documentation system; utilizing Gensnap software, Syngene (Frederick, MD, USA). Profiles were scored as genetic data on the basis of presence or absence of fragments presumed to be the same length and same size. From these data, a binary matrix was generated. ERIC rep-primers did not amplify *P. griseola* isolates from DRC; data from ERIC rep-primers was not considered in analysis. The data from REP and those from BOXAIR-PCR primer were combined as one set of data. The evolutionary analysis was conducted by MEGA 5 (Tamura et al., 2011). Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA). Booststrap value was based on 1000 permutations of the data and presented in percentages.

The analyses of H_S, G_{ST}, and Nei's genetic identity and genetic distance between populations were performed using the computer software POPGENE (version 1.31, University of Alberta, Edmonton, Canada). Gene diversity between subpopulations is given by the difference in gene diversity between the total population and within the subpopulations (H_S), thus: $D_{ST} = H_T - H_S$ (Nei, 1973). Population differentiation (G_{ST}) was calculated from gene diversity between subpopulations (D_{ST}) and gene diversity in the total population (H_T) as given by $G_{ST} = D_{ST}/ H_T$. Gene flow between populations (Nm), where N is the population size and m is the fraction of individuals in a population that are migrants, was estimated by substituting Nei's G_{ST} for F_{ST} in Wright's model of gene flow (Wright, 1950) using a corrected version for haploids, thus: $Nm = 0.5[(1 - G_{ST})/G_{ST}]$ (McDermott and McDonald, 1993). If $Nm<1$, then local populations tend to differentiate; if $Nm\geq1$, then there will be little differentiation among populations and migration is more important than genetic drift (Wright, 1950). A graphical representation of genetic similarities among *P. griseola* was obtained by generating a dendogram from the similarity matrices and the UPGMA clustering method. For more than 340 isolates collected during the survey, 80 isolates from middle altitude (MA) and high altitude (HL) succeeded (Table 1). Of these, 59 were selected for pathogenicity test (Table 3) and 36 that got good DNA were used for molecular characterization, based on their host genotypes and geographic origin. Leaf area damage thresholds depicted on a visual scale were used to classify symptom ratings into compatible and incompatible reactions (Schoonhoven and Pastor-Corrales, 1987). Pathotype designation was based on the sum (binary values) of bean differential cultivars with ratings of >3 (compatible reaction) and <3 (incompatible reaction) (Mahuku et al., 2002a).

RESULTS

Virulence of the DRC *P. griseola* isolates

High diversity was observed among ALS differentials cultivars regarding their reaction to the different *P. griseola* isolates. Over eighty percent of the bean differentials were susceptible. No differential genotype showed resistant reaction to all the isolates.

Among the differential varieties, Mexico 54 showed resistant reaction to most of the isolates, except nine of them, such as: SK176,SK 177, SK 178, NK 209, SK 214, SK 223, SK 246, SK 289 and SK 311, to which it showed intermediate reaction (4-6), on the basis of 1-9 CIAT standard scale. Differential cultivar G5686 revealed also resistant reaction to 84.7% of the isolates.

Of the 59 isolates tested for pathogenicity, only three isolates, SK 161 (5-0), SK 241 (1-0), NK 69 (57-0) attacked exclusively Andean genotypes, while three SK152 (0-8), SK 177 (0-12), SK 178 (0-12) infected exclusively Mesoamerican genotypes. The rest attacked both Andean and Mesoamerican genotypes. Pathotypic diversity was high in the highlands agroecological zone, where different isolates were grouped into similar pathotype, e.g. Pathothype 21-39 (7.7%) of isolates. The most virulent isolates were also recovered in the same agroecological zone (South Kivu) as compared to mid-altitudes zone (North Kivu). Pathotype 61-55 (SK 210) infected most of both Andean and Mesoamerican cultivars, whereas pathotype 61-39 (SK 126) infected most of the Andean bean cultivars, and pathotype 5-62(SK 176) infected most of the Mesoamerican bean cultivars. However, there was Mesoamerican isolates that infected also some Andean differential cultivars. 12 isolates were classified afro-andean, 29 mesoamerican,

Table 1. List of isolates used and collection sites.

No.	Isolate code/ID	Agro-ecological zones	Location/District	Host genepool	Collection year and month	Altitude (masl)
01	NK 01	MA	Lubero	Meso	7/2011	1931
02	NK 10	MA	Lubero	Andean	7/2011	1866
03	NK 12	MA	Lubero	Meso	7/2011	2110
04	NK 13	MA	Lubero	Andean	7/2011	1866
05	NK 15	MA	Lubero	Meso	7/2011	2110
06	NK 17	MA	Lubero	Meso	7/2011	2148
07	NK 26	MA	Lubero	Meso	7/2011	1737
08	NK 34	MA	Lubero	Meso	7/2011	1874
09	NK 36	MA	Lubero	Meso	7/2011	2168
10	NK 38	MA	Lubero	Meso	7/2011	1736
11	Nk 39	MA	Lubero	Andean	27/011	1736
12	NK 42	MA	Masisi	Andean	7/2011	1750
13	NK 54	MA	Masisi	Meso	7/2011	1573
14	NK 49	MA	Masisi	Andean	7/2011	1488
15	NK 57	MA	Masisi	Meso	7/2011	1485
16	NK 61	MA	Masisi	Meso	7/2011	1495
17	NK 63	MA	Masisi	Andean	7/2011	1736
17	NK 69	MA				
18	NK 71	MA	Rusthuru	Andean	7/2011	1482
19	NK 80	MA	Rusthuru	Anden	7/2011	1573
20	NK 104	MA	Rusthuru	Meso	7/2011	1172
21	NK 111	MA	Rusthuru	Andean	7/2011	1205
22	NK 112	MA	Rusthuru	Meso	7/2011	1205
23	NK 200	MA	Rusthuru	Andean	11/2012	1881
24	NK 209	MA	Rusthuru	Andean	11/2012	1656
25	NK 189	MA	Rusthuru		11/2012	1801
26	NK 190	MA	Rusthuru		11/2012	1724
27	NK 191	MA	Rusthuru	Meso	11/2012	1768
28	NK 192	MA	Rusthuru	Meso	11/2012	1729
29	NK 185	MA	Rusthuru		11/2012	
30	SK 43	HL	Kabare	Meso	7/2011	1730
31	SK 45	HL	Kabare	Andean	7/2011	1730
32	SK 126	HL	Kabare	Andean	11/2012	1497
33	SK 146	HL	Kabare	Meso	11/2012	1536
34	SK 151	HL	Uvira	Andean	11/2012	878
35	SK 152	HL	Uvira	Meso	11/2012	881
36	SK 154	HL	Uvira	Andean	11/2012	881
37	SK 157	HL	Uvira	Meso	11/2012	894
38	SK 160	HL	Uvira	Andean	11/2012	905
39	SK 161	HL	Uvira	Andean	11/2012	900
40	SK 163	HL	Uvira	Meso	11/2012	972
41	SK 175	HL	Walungu	Meso	11/2012	1636
42	SK 176	HL	Walungu	Meso	11/2012	1579
43	SK 177	HL	Walungu	Andean	11/2012	1561
44	SK 178	HL	Walungu	Meso	11/2012	1536
45	SK 182	HL	Walungu	Andean	11/2012	1430
46	SK 184	HL	Walungu	Meso	11/2012	
47	SK 210	HL	Kabare	Andean	12/2012	1671
48	SK 211	HL	Kabare	Andean	122012	1671
49	SK 212	HL	Kabare	Meso	12/2012	1610

Table 1. Contd.

50	SK 214	HL	Kabare	Andean	12/2012	1618
51	SK 219	HL	Kabare	Meso	12/2012	1704
52	SK 222	HL	Kabare	Andean	12/2012	1582
53	SK 223	HL	Kabare	Meso	12/2012	1620
54	SK 224	HL	Kabare	Andean	12/2012	1620
55	SK 226	HL	Kabare	Meso	12/2012	1588
56	SK 232	HL	Kabare	Andean	12/2012	1635
57	SK 234	HL	Kabare	Meso	12/2012	1573
58	SK 237	HL	Kabare	Meso	12/2012	1564
59	SK 239	HL	Kabare	Meso12/	12/2012	1473
60	SK 240	HL	Kabare	Meso12/	12/2012	1503
61	SK 241	HL	Kabare	Andean	12/2012	1503
62	SK 243	HL	Kabare	Meso	12/2012	1486
63	SK 246	HL	Kabare	Meso	12/2012	1707
64	SK 247	HL	Kabare	Andean	12/2012	1707
65	SK 249	HL	Kabare	Meso	12/2012	1735
66	SK 274	HL	Uvira	Andean	12/2012	1152
67	SK 276	HL	Uvira	Meso	12/2012	1064
68	SK 279	HL	Uvira	Meso	122012	891
69	SK 280	HL	Uvira	Anden	12/2012	951
70	SK 281	HL	Uvira	Andean	12/2012	
71	SK 282	HL	Uvira	Meso	12/2012	
72	SK 288	HL	Uvira	Andean	12/2012	942
73	SK 291	HL	Kabare	Andean	12/2012	2086
74	SK 298	HL	Kabare	Meso	12/2012	1733
75	SK 302	HL	Kabare	Meso	12/2012	1751
76	SK 311	HL	Kabare	Meso	12/2012	1679
77	SK 313	HL	Kabare	Andean	12/2012	1714
78	SK 289	HL	Uvira		12/2012	

Table 2. Sequences and annealing temperatures of repetitive primers.

Primers	Primers sequences (5'-3')	Annealing temperature (°C)
BOX A1R	5' CTA CGG CAA GGC GAC GCT GAC G3'	40
BOX1 B	5TTCGTCAGTTCTATATCTACAACC-3'	40
REP IR	5'-IIIICGICGICATCIGGC-3'	40
REP1RDt	5'-IIINCGNCGNCATCNGGC-3'	40
REP2.Dt	5'-NCGNCTTATCNGGCCTAC-3'	40
REP 2I	5'-ICGICTTATCIGGCCTAC-3'	40
ERIC IR	5'ATGTAAGCTCCTGGGGATTCAC-3'	52
ERIC2I	5'AAGTAAGTGACTGGGGT GAGCG-3'	52

Source: Versalovic et al. (1994).

Analysis of variance revealed highly significant differences (P<0.001) between differential varieties regarding their reaction to ALS isolates. There was highly significant interactions between isolates and varieties (P<0.001).

Genetic structure

Repetitive element REP-PCR and BOXAIR primers selected for this study generated 45 strong bands/loci that were scored unambiguously. Presence or absence of

Table 3. Reactions of differential cultivar and pathotypes of *P. griseola* from eastern DRC.

Isolate	Reaction of ALS differentials to *P. griseola*												Pathotypes	Virulent group	Origin genotype
	Andean						Mesoamerican								
	A	B*	C	D	E	F	G	H	I	J	K	L			
	Binary value														
	1	2	4	8	16	32	1	2	4	8	16	32			
NK 13	+		-	+	-	+	+	+	+	-	+	+	41-55	Afro	A
NK 36	+		-	-	-	-	+	+	+	-	-	+	1-39	Meso	M
NK 38	+		-	-	-	-	-	+	+	-	-	-	1-8	Meso	M
NK 50	+		+	-	-	-	+	+	+	-	-	+	5-39	Meso	M
NK 54	+		-	-	+	-	-	+	+	-	+	-	17-22	Meso	M
NK 111	+		-	+	-	+	-	+	+	-	-	-	41-6	Andean	A
NK 10	+		-	+	-	-	+	+	+	-	-	+	9-39	Meso	M
NK 12	+		+	+	+	-	+	+	+	-	+	+	29-55	Meso	M
NK 15	+		+	+	+	-	+	+	+	-	+	-	29-55	Meso	M
NK 39	+		+	+	-	-	+	+	+	-	-	-	13-39	Afro	A
NK 42	+		+	-	-	-	+	+	+	-	-	-	5-23	Afro	A
SK 43	+		+	+	-	-	+	+	+	-	-	+	13-39	Afro	M
SK 45	+		+	+	-	-	-	+	+	-	+	-	13-22	Andean	A
NK63	+		-	+	-	-	+	+	+	-	-	-	9-23	Meso	M
NK 69	+		-	+	+	+	-	-	-	-	-	-	57-0	Andean	A
NK 80	+		+	+	-	-	+	+	+	-	+	+	13-55	Afro	A
NK 1	+		-	-	+	-	+	+	+	-	-	-	17-39	Meso	M
NK 17	-		-	-	-	-	-	-	-	-	-	-	0-0		M
NK 26	-		-	-	-	-	-	-	-	-	-	-	0-0		M
NK 34	-		-	-	-	-	-	-	-	-	-	-	0-0		M
NK 61	+		+	-	+	-	+	+	+	-	-	+	21-39	Meso	M
NK 104	-		-	-	-	-	-	-	-	-	-	-	0-0		M
SK 151	-		-	+	-	+	+	+	+	-	-	-	40-7	Andean	A
SK 152	-		-	-	-	-	-	-	+	-	-	-	0-8	Meso	M
SK 154	+		+	-	+	-	+	+	+	-	-	+	21-39	Afro	A
SK 157	-		-	-	-	-	-	-	-	-	-	-	0-0		M
SK 160	-		-	+	+	-	-	-	+	-	-	-	24-4	Andean	A
SK 161	+		+	-	-	-	-	-	-	-	-	-	5-0	Andean	A
SK 163	-		-	+	-	+	+	+	+	-	-	-	40-7	Andean	A
SK 175	-		-	-	-	-	-	-	-	-	-	-	0-0		M
SK 176	+		+	-	-	-	-	+	+	+	+	+	5-62	Meso	M
SK 177	-		-	-	-	-	-	-	+	+	-	-	0-12	Meso	M
SK 178	-		-	-	-	-	-	-	+	+	-	-	0-12	Meso	M
SK 182	+		+	-	+	-	+	+	+	-	-	+	21-39	Afro	A
SK 184	+		-	-	-	-	-	+	+	-	-	-	1-6	Meso	M
SK 126	+		+	+	+	+	+	+	+	-	-	+	61-39	Afro	A
SK 146	+		-	+	+	+	+	+	+	-	-	-	57-39	Meso	M
NK 200	+		+	+	+	-	+	+	+	-	+	+	29-55	Afro	A
NK 209	+		+	+	-	-	+	+	+	+	-	-	13-15	Afro	A
NK 224	+		+	-	-	-	+	-	-	-	+	-	5-22	Meso	M
SK 211	+		+	+	+	+	-	+	-	-	+	-	61-20	Andean	A
SK 222	+		-	-	-	-	+	+	+	-	+	+	1-55	Meso	M
SK 232	-		-	-	-	-	-	-	-	-	-	-	0-0		A
SK 214	+		-	+	+	-	-	-	-	+	-	-	25-8	Andean	A
SK 223	+		-	+	+	+	-	+	+	+	-	-	29-39	Meso	M
SK 241	+		-	-	-	-	-	-	-	-	-	-	1-0	Andean	A

Table 3. Contd.

SK 243	+	+	-	-	-	+	+	+	-	-	+	5-55	Meso	M
SK 247	+	+	-	-	-	+	+	+	-	+	-	5-23	Afro	A
SK 249	+	+	-	-	-	+	+	+	-	-	-	5-39	Meso	M
SK 212	+	+	+	-	-	+	+	+	-	-	+	13-39	Meso	M
SK 210	+	+	+	+	+	+	+	+	-	+	+	61-55	Afro	A
SK 237	+	+	-	+	-	+	+	+	-	-	+	21-39	Meso	M
SK 239	+	-	-	-	-	+	+	+	-	+	+	1-55	Meso	M
SK 240	-	-	-	+	-	+	+	-	-	+	-	16-22	Afro	A
SK 246	+	-	-	+	-	+	+	-	+	+	+	25-55	Meso	M
SK 282	+	+	+	-	-	-	+	+	-	+	+	13-55	Meso	M
SK 289	+	-	-	-	-	-	+	-	+	+	+	1-58	Meso	M
SK 311	+	+	+	-	-	+	+	+	-	+	+	13-55	Meso	M
SK 219	-	-	-	-	-	-	-	-	-	-	-	0-0		M

Differentials cultivars are designated by alphabetic letters: A up to F are Andean genotypes: A. Don Timeteo, B. G11796, C. Bolon Bayo, D. Montcalm, E. Amendoin, F. G5686. G up to L are Mesoamerican genotypes: G. Pan 72, H. G2856, I. Flor de Mayo, J. Mexico 54, K. Bat 322, L. Cornell 49242. Pathotypes designation is based on the sum (binary values) of bean cultivars with > 3 scale value. (+). Compatible reaction; (-) Incompatible reaction. All pathogenicity tests included three replicates per isolate. NK: North Kivu, SK: South Kivu A: Andean M: Mesoamerican.*. Differential cultivar B=G11796, with blank column, did not produce seeds under Ugandan condition.

A high level of genetic diversity was found when isolates from farmers' fields at different agroecological zones were evaluated. The repetitive element primers identified 27 and 42 polymorphic loci in sub-humid mid-altitude (MA) and sub-humid highlands (HL) *P. griseola* populations, respectively. Average gene diversity across all loci was high for both agroecologies (H_T=0.31) (Table 4), with moderate genetic differentiation (Gst=19%) of *P. griseola* population in the two agro-ecological zones. However, the low average gene diversity between sub-population from the two agro ecological zones (D_{ST}= 0.06 of the total gene diversity) indicated that most of the genetic diversity was found within each subpopulation, with little diversity between subpopulations.

Evaluation of genetic diversity of *P. griseola* isolates from different genotypes (large seeded or Andean and small seeded or Mesoamerican) revealed that an average gene diversity was moderately high (Hs=0.27) indicating that most of the genetic diversity was found within the subpopulations (96%) with only 4%, accounted for difference between populations from different host genotypes. However, a moderate genetic differentiation G_{ST}=12% of *P. griseola* population from bean of both host genotypes was discerned. With Nei's measures of genetic identity I=0.90%, this indicates a 90% chance of choosing the same allele at any locus in a random draw of two individuals from these populations. A graphical representation of genetic similarities among *P. griseola* was obtained by generating a dendogram from the similarity matrices based on the UPGMA clustering method. This allowed the differentiation of eastern DRC *P. griseola* populations into two main clusters (1 and 2) at 65% genetic similarity, based on the combined REP and BOXAIR dataset (Figure 2). Among the two main clusters revealed by DNA fingerprinting, the minor cluster (1) was composed exclusively of isolates from highlands area (HL) from South Kivu. This was divided into two sub-clusters (A and B) with the sub-cluster (B) grouping isolates strictly from small seeded (Mesoamerican) and sub-cluster (A) clustering isolates from large seeded (Andean). These sub-clusters (A) and (B) were 80% genetically similar. The main cluster (2) grouping many isolates (Figure 3), this was divided into three sub-clusters: sub-cluster (A) composed of isolates from highlands area (South Kivu) collected from large seeded, sub cluster (B) comprised isolates from mid-altitudes (North Kivu) and others from highlands (South Kivu), sub-cluster (C) composed only with isolates from mid-altitudes area (MA), North Kivu. These sub-clusters were 86% genetically similar with isolates from Andean or Mesoamerican host genotypes clustering together. The dendogram showed a trend of North Kivu isolates to form a sub-cluster within South Kivu isolates. However, genetic study of *P. griseola* isolates from DRC did not elucidate an Afro Andean sub-cluster.

DISCUSSION

Virulence of the DRC *P. griseola* isolates

Among the differential varieties, Mexico 54 showed resistant reaction to most of the 59 isolates, except nine of them to which it showed intermediate reaction 4-6, based on CIAT scale 1-9. This variety can serve as source of resistance to improve common bean resistance

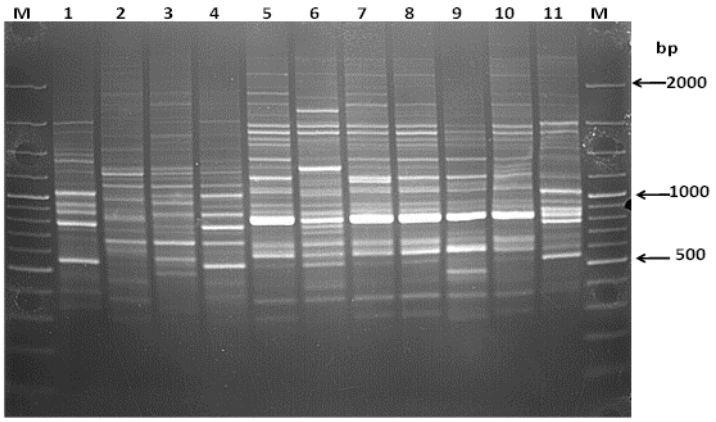

Figure 2. Repesentative of gel photo using BOXIR-PCR primers. M both sides represent 100bp molecular marker: Lines 1-5 *P. griseola* isolates collected on bean in North Kivu: 1. NK 1, 2. NK13,3. NK15, 4. NK 33, 5. NK 80, Lines 6-11 represent *P. griseola* isolates collected on bean in South Kivu: 6. SK 126, 7. SK 161, 8. SK 289, 9. SK 175, 10. SK 302, 11. SK 313.

to ALS, in DRC. Mexico 54 was also found as Mesoamerican differential to be resistant to the ALS disease in Brasil (Sartorato and Alzate-Marin, 2004). Mexico 54 has shown low frequency percentage of pathogen infection and has been suggested as parental lines with potential source of resistance gene in Ethiopia (Rezene et al., 2018). This variety Mexico 54 was found to be resistant to most races of *P. griseola* and among highly resistant source to ALS in breeding in Uganda, East Africa and elsewhere (Njoki, 2013; Nay et al., 2019; Rodríguez et al., 2019). Differential cultivar G5686 revealed also resistant reaction to 84.7% of the isolates. This differential cultivar among others such as Amendoin and Mexico 54 exhibited the lowest scores for the severity to angular leaf spot in Brazil (Pereira et al., 2015).

These pathotypes SK 126 (61-39), SK 176 (5-62) and SK 210 (61-55), that have shown complex virulence capability, affecting either most of the andean cultivars, or mesoamerican cultivars or both could be the most appropriate for screening bean for ALS resistance and identification of resistant varieties in eastern DRC. However, race (63-63) is able to overcome all differential cultivars was identified as the most aggressive and frequent in Brasil and was used to screen bean germoplasm, for resistance to ALS identification (Pádua et al., 2022). The high number of pathotypes discerned 35 pathotypes over 59 isolates and the presence of isolates is able to infect similar host genotypes (Andean and Mesoamerican), where they have been collected confirm the high pathogenic variability and the specialization to host genotypes as reported by Mahuku et al. (2002a), as well as the possibility of *P. griseola* population, from DRC to be broadly grouped into two structured pathogenic groups (Mahuku et al., 2002a). Previous study by Pyndji (1992) identified 17 pathotypes among 21 isolates from DRC, Rwanda and Burundi, this confirmed also the high pathogenic diversity in the Grands Lacs Region, while in Kenya, 44 races were found among one hundred isolates based on a set of differential cultivars (Wagara et al., 2004), however, 21 pathotypes were identified among 39 occuring isolates in Ethiopia (Rezene et al., 2018). Fifty nine isolates from DRC used for pathogeneicity study were classified into 3 virulence group, such as Andean, Afro-Andean and Mesoamerican. Conversely, isolates from Kenya were also divided into 3 virulence groups, comprising Andean, Afro-Andean and Mesoamerican (Wagara et al., 2004). Furthermore, seven pathotypes were identifed from fifty one isolates from Brazil as mentionned by Sartorato (2002).

Genetic structure

A total of 45 bands were amplified by the repetitive elements: PCR primers, REP and BOXA IR, used. Among the 45 loci, 43 were polymorphic in all population examined. The high level of polymorphism (on average 95.5%) revealed by REP and BOXAIR primers indicated that the markers are highly valuable to be used in subsequent diversity studies. Our findings are in agreement with the previous authors who reported the efficiency of these repetitive elements as PCR based markers (Dawson et al., 2002; Muiru et al., 2010; Sari et al., 2010; Çepni and Gürel, 2012).

Although, cluster analysis showed that subpopulations from sub-humid highlands and sub-humid mid-altitudes areas were closely related, the moderate genetic differentiation (G_{ST}= 0.19) that was observed between *P.*

Table 4. Genetic diversity between and within *P. griseola* populations based on REP and BOXAIR-PCR analysis.

Population	N	NPL[a]	% PL[b]	H_T[c]	H_S[d]	D_{ST}[e]	G_{ST}[f]	Nm[g]	I[h]	D[i]
Sub-humid mid-altitude(MA)	12	27	60							
Sub-humid highlands(HL)	24	42	93.3							
MA vs HL	36	43	95.6	0.31	0.25	0.06	0.19	2.10	0.85	0.15
Small-seeded (meso)	17	34	75.56							
Large-seeded (andean)	19	42	93.33							
Small vs. large types	36	43	95.56	0.31	0.27	0.04	0.12	3.4	0.90	0.1
Low altitude	7	30	66.66							
High altitude	29	43	95.96							
Low vs. high altitudes	36	43	95.56	0.29	0.28.	0.01	0.03	15.0	0.99	0.01

[a]NPL= number of polymorphic loci; [b] %PL= percentage of polymorphic loci; [c]H_T=total gene diversity; [d]HS= within subpopulation gene diversity; [e] D_{ST}= between sub-population gene diversity; [f]G_{ST}=genetic differentiation; [g]*Nm* = gene flow; [h] *I*=genetic identity; [i]*D* = genetic distance, N=Number.

griseola populations from the two agro-ecological zones, indicated the possible influence of agro-ecology on the structure of *P. griseola* in eastern DRC. This breeding for ALS resistence in DRC should take care of existing sub group and different agroecologies.

This limited differentiation between geographical *P. griseola* sub-populations could be due to the fact that, INERA-Mulungu Research Centre, which is located in the highland areas of South Kivu provides most of the basic seed used for adaptive testing and large-scale seed production by diverse partners in the mid-altitude areas of North Kivu. In addition, mid-altitude areas in North Kivu having more fertile soils that constitute the main bean production area, from where beans are marketed to other provinces in the country (including South Kivu and neighbouring countries). It is possible that this germplasm exchange and extensive bean trade has facilitated gene/genotype flow leading to limited differentiation between geographic sub-populations. Abang et al. (2006) also attributed the genetic similarity of geographic populations of *Colletotrichum gloeosporioides* from *Dioscorea alata* (yam) in Nigeria to the yam trade that takes place across the country. Similar findings were reported by Bouajila et al. (2007). On the other hand, 82 MaYMV sequences clustered into three phylogenetic groups that were associated with their geographical origins (China, Africa and South America) to some extent (Sun et al., 2021).

The high genetic similarity indices among the isolates in different clusters on the dendogram indicates that isolates were closely related (Figure 3). This suggests that such isolates have a familial structure or are clonally related (Kiros-Meles et al., 2005). *P. griseola* obtained from same altitude area did not cluster together on the dendogram, indicating that the occurrence of specific *P. griseola* associated with distinct altitude was less supported by analysis of gene diversity and genetic differentiation (*Gst*< 0.03). Consequently, there is no evidence that there may be a significant barrier to gene flow operating in major common bean growing areas of eastern DRC. Analysis of *P. griseola* populations from different host genotypes revealed a moderate genetic differentiation (G_{st}=12%), which indicated a moderate level of differentiation between *P. griseola* isolates obtained from Andean and Mesoamerican bean genotypes in this study. However, coefficient of genetic differentiation (Gst=0.27) observed for *Puccinia triticina* in Hebei province of China indicated that there was a genetic differentiation among or within populations (Zhang et al., 2023). Nevertheless, Nei's measures of genetic identity, I=0.90%, this indicates a 90% chance of choosing the same allele at any locus in a random draw of two individuals from which these population were observed.

In a similar study, Mahuku et al. (2002a) reported a significant level of genetic differentiation (Gst=0.57) between Andean and Mesoamarican isolates showing the strong influence of host genotype on the pathogen population structure. ISSR analysis of *P. griseola* isolates from Argentina revealed 17 haplotypes that were grouped into two main clusters with 56% genetic dissimilarity (Stenglein and Balatti, 2006). Genetic study of *P. griseola* isolates from DRC did not elucidate an Afro Andean Cluster, even in the previous study by Mahuku et al. (2002a), few isolates from DRC (4) included in this study, none of them fallen in Afro-Andean sub-cluster.

This study confirms that *P. griseola* population in DRC tends to be broadly grouped into Andean and Mesoamerican, corresponding to their respective host genotypes, a finding which agrees with the previous reports on co-evolution of *P. griseola* with its host (Pastor-Corrales et al., 1998; Mahuku et al., 2002a; Aggarwal et al., 2004; Stenglein and Balatti, 2006).

However, our conclusion should be treated with some caution because of the relatively small samples size (especially from North Kivu) and the limited area sampled. However, bootstrap tests of significance enabled us to estimate indices of diversity and population

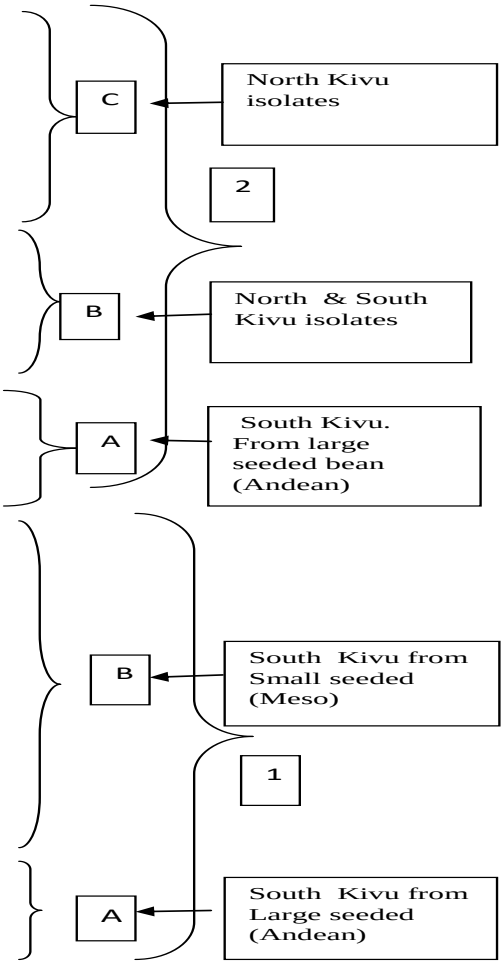

Figure 3. Figure dendogram mixed data REP and BOX primers. Dendrogram illustrating relationships among *P. griseola* isolates sampled from common bean in eastern DRC based on Rep-PCR fingerprinting. Isolates are designed by alphanumerics indicating the province (NK and SK representing North Kivu and South Kivu) followed by isolate number, e.g. 10 for isolate identity, and two letters (HL and MA indicating highland and mid-altitude agro-ecological zones), then numbers separated by dash representing pathotypes (e.g. 9-39), followed by a letter at the end representing grain size (L and S for large-seeded and small-seeded). 1. Numbers on dendogram branches indicate bootstraps values. 2. Scale down of the dendogram represent indices of dissimilarities.0.0 correspond to 100% genetically identical.

differentiation with a reasonable degree of confidence.

The origin of the high molecular and pathogenic diversity in *P. griseola* is unclear as this fungus has no known sexual cycle. Evidence that high levels of haplotypic diversity can be maintained in asexually reproducing fungi through parasexual reproduction has been reported (Stenglein and Balatti, 2006; Scheuermann et al., 2012). Mahuku et al. (2002a) reported that multiple infections by *P. griseola* belonging to different groups might lead to parasexual recombination. Other mechanisms such as mutations, gene/genotypes flow followed by host selection and adaptation to different environments could explain the high diversity found in the *P. griseola* population in DRC. Many farmers in eastern DRC grow beans as cultivar mixtures, which might favour high pathogen diversity. Sustainable management of crop pathogenes must include optimized agricultural practices, including molecular approaches aiming for early detection effective crop protection (Santos and Silva, 2023). Recent studies have shown that even though one-fifth of described fungi are thought to be asexual and clonal, they are also recombining, depending on factors relating to their biology and distribution in space and time (Taylor et al., 1999).

CONCLUSION AND RECOMMENDATIONS

Thirty five pathotypes (races) were identified among the 59 isolates used for virulence test. Pathotypic diversity was high in the highlands agroecological zone. The most virulent isolates were also recovered in the same agroecological zone (South Kivu), compared to mid-altitudes zone (North Kivu). These pathotypes SK 126 (61-39), SK 210 (61-55) and K 176 (5-62) with complex virulence capability could be the most appropriate for screening bean for ALS resistance and identifying of ALS resistant varieties in eastern DRC. Mexico 54 that have shown resistance reaction to most of the DRC isolates could be used as source of resistance to ALS disease by breeding program in DRC. The *P. griseola* population from eastern DRC is characterized by high level of DNA polymorphism, with moderate levels of gene diversity and genetic differentiation. The high genetic similarity indices among isolates in different clusters on the dendogram indicated that such isolates have a familial structure or are clonally related. *P. griseola* population in DRC tend to be broadly grouped into Andean (16%) and Mesoamerican (49%), corresponding to their respective host genotypes.

This high variability needs more attention, while developing bean populations with durable resistance to angular leaf spot. The findings of this study represent a useful guide for the breeding program in eastern DRC.

Evaluation of genetic diversity of *P. griseola* isolates from different genotypes (large seeded or Andean and small seeded or Mesoamerican) revealed that an average gene diversity was moderately high (Hs=0.27) indicating that most of the genetic diversity was found within the subpopulations (96%) with only 4% accounted for between and with *P. griseola* populations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank CIAT/PABRA for funding this study, colleagues from CIAT/PABRA-Uganda, and INERA Mulungu research station, in DRC for their technical support.

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