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Pathogen Variability and New Sources of Resistance to Angular Leaf Spot among Bean Landraces in Uganda

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Abstract
Angular leaf spot (ALS), a fungal disease caused by *Pseudocercospora griseola,* has a significant impact on bean productivity in Africa, causing up to 80% yield losses. Efforts to breed for ALS resistance are challenged by continuously evolving pathogen races that differ by location. Released bean varieties in Uganda are susceptible to ALS; the few exotic sources of resistance available are not well adapted to local environmental conditions. To overcome these challenges, a study was conducted to understand pathogen variability and identify new sources of ALS resistance for deployment in ALS resistance breeding. Variability in 45 *P. griseola* pathotypes was elucidated using a set of 12 ALS differential cultivars, random amplified microsatellite markers, and conserved sequences. The differentials and markers defined 12 pathotypes and 30 haplotypes, respectively, which belonged to the Middle American and Andean gene pool groups, each with high variability. Among the 74 bean landraces screened using the 1:6, 17:39, 21:39, and 61:63 *P. griseola* pathotypes, only U00279 showed consistent resistance to all the four pathotypes. U00297's resistance to pathotype 17:39 was conferred by a single dominant gene, while digenic epistatic gene interactions were responsible for resistance to other pathotypes. The dominant gene in U00297 was independent of resistance genes harbored by documented resistance sources AND277 and G5686. The results revealed high variability in *P. griseola* and identified a new source of broad ALS resistance. The divergent inheritance patterns of resistance to the different pathotypes indicate the importance of race specificity of the target host plant in breeding for disease resistance.

Key words: *Pseudocercospora griseola*, variability, resistance, inheritance, allelic

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Introduction

Angular leaf spot (ALS) is among the most destructive diseases of common bean (*Phaseolus vulgaris*) in tropical and subtropical regions (Allorent and Savary, 2005). The disease is ranked second among the biotic factors that constrain bean production in Africa (Wangara *et al*., 2003). In Eastern Africa, where beans are commonly grown and consumed by most households, 3,961,679 MT are produced annually (FAO, 2012) of which 374,800 MT are lost due to ALS (Wangara *et al*., 2003). In Uganda, yield losses of up to 50% have been reported in commercial varieties (Opio *et al*., 2001). ALS infection on leaves and stems results in premature defoliation, shriveled pods and shrunken seeds, hence, reducing the yield potential of beans (Strenglein *et al*., 2003). In addition, late infection on pods and seeds also causes scars that reduces seed quality and market value (Mahuku *et al*., 2003).

The widespread distribution of ALS in Uganda and within the Great Lakes region is mainly attributed to the use of susceptible cultivars and uncertified seed (Wagara *et al*., 2003). Furthermore, the intermittent dry-wet and warm-cool weather which characterize weather conditions in most bean growing areas also accelerate *P. griseola* sporulation process (Celleti, 2006), which encourage pathogen proliferation leading to heavy infestation. Furthermore, control of ALS remains difficult due to the high pathogenic variation of the fungus (Mahuku *et al*., 2004). Based on morphological and molecular markers, two gene pools of origin for common bean have been defined, namely; the Andean and Middle American. The Andean isolates are pathogenic to large seeded beans, while the Middle Americans are pathogenic to both small and large seeded beans (Guzmán *et al*. 1995; Pastor-Corrales *et al*. 1998). Earlier studies have also indicated that many races of *P. griseola* occur and vary in time and space; a bean cultivar which is resistant in one location, season or year may be susceptible in another (Aggarwal *et al*., 2004). For this reason, understanding *P. griseola* variability is critical in designing control strategies for ALS, but this remains unknown in Uganda and, probably as a consequence, there is hardly any commercial cultivar either tolerant or resistant that has been developed.

In the smallholder farming systems of Uganda, ALS is currently managed through cultural practices such as crop rotation and cultivar mixtures. However, these have limited potential in managing the disease, because land scarcity cannot allow crop rotation to be practiced (Stenglein *et al*., 2003). Besides, effective ALS control methods like use of fungicide are not affordable for smallholder farmers because of the high cost and long-term negative consequences fungicide pose to human health and the environment (Mahuku, 2002). In such a situation, use of genetic resistance is the most appropriate, safe and cost-effective way to control ALS in smallholder farming systems (Wagara *et al*., 2003).

A number of exotic sources of ALS resistance do exist and have been utilized in breeding programs targeting ALS; they include, among others, Mexico 54, MAR1, MAR2, AND277, G5686, G10909, and G10474 (Mahuku *et al*., 2003, Caixeta *et al*., 2005). Their limitations are low adaptability and undesirable traits. Most resistant sources are adapted to environments in which they originated or were developed; this limits their use in other environments where they are not adapted to (Holbrook *et al*.,

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2000). Besides, the majority of resistance sources are small-seeded with a climbing growth pattern; such attributes are not readily accepted by farmers in Uganda, and Africa at large (Beebe *et al*., 1981).

However, landraces maintained by farmers have for a long time been known to have useful agronomic traits. Indeed, most existing resistance sources developed elsewhere have been derived from landraces (Busogoro *et al*., 1999). For instance, G5686, which is a good source of ALS resistance and a member of the ALS differential set, is a landrace that originated from Ecuador (Mahuku *et al*., 2009). Though resistance may exist in some landraces, the high degree of genetic variability of *P. griseola* often compromises the use of ALS resistance derived from landraces (Nietsche *et al*., 2001). This is due to continuous emergence of new races, which break down disease resistance (Young *et al*., 1998). Hence, the need for continuous screening of germplasm to identify new sources of resistance that can regularly be introgressed into commercial cultivars (Young and Kelly, 1996). This will counteract the new emerging races and reinforce resistance in existing ALS resistance sources.

Nonetheless, identifying new sources of resistance alone cannot guarantee full protection of beans against ALS since resistance often breaks down (McDermott, 1993). Moreover, due to high *P. griseola* pathogenic variability, no known single resistance gene has been reported to be effective against all races. One way of developing stable resistance against such a variable pathogen, is by pyramiding several resistance genes into a single genotype with desirable traits. But pyramiding depends heavily on information related to inheritance and allelic relationship between resistant sources (Namayanja *et al*., 2006). Therefore, the study aimed at determining the variability of *P. griseola* in Uganda; identify new, adapted sources of ALS resistance, and the mode of inheritance as well as the allelic relationship between the identified new and existing sources of resistance.

Materials and methods

Fungal Isolation and Inoculum Preparation

Forty-five single spores *P. griseola* isolates previously obtained from major bean growing districts of Uganda were activated to elucidate the relationship between isolates belonging to the Middle American and Andean pools. Activation, monosporic culture production and inoculums preparation were done according to Pastor-Corrales *et al*. (1998). Spore concentration in the inocul*ums* was estimated using a haemocytometer and adjusted to a final concentration of 2 x 10^4 conidia ml^{-1} using sterile distilled water.

Inoculation and Pathotype Determination

In order to determine the pathotype of each isolate, a set of 12 differential cultivars consisting of six Andean (DonTimoteo, G 11796, Bolon Bayo, Montcalm, Amendoin, G 5686) and six Mesoamerica (Pan 72, G 2858, Flor deMayo, Mexico 54, BAT 332, Cornell 49–242) cultivars were used. Five seeds of each differential cultivar were planted in 5-litre buckets containing black soil, manure and sand in a ratio of 3:1:1. The experiment was laid out in completely randomized design with three replications. The 21-day-old bean plants were spray-inoculated with inoculums and kept in a humid

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chamber at 22-28 °C and 95% relative humidity. Four days after inoculation, plants were transferred into the screen house and watered regularly.

Disease symptoms on the inoculated plants were evaluated using a CIAT 1-9 visual scale (Schoonhoven and Pastor-Corrales, 1987) for 21 days at an interval of three days, described as follows: 1, plants with no visible disease symptoms; 3, presence of a few small non-sporulating lesions that cover approximately 2% of the leaf surface; 5, plants with several small lesions with limited sporulation and covering approximately 5% of leaf surface; 7, plants with abundant and generally large sporulating lesions covering approximately 10% of leaf surface and associated with chlorosis and necrosis; and 9, 25% or more of leaf surface with large sporulating and often coalescing lesions, frequently associated with chlorosis resulting in severe and premature defoliation. Pathotypes were defined by rating scores of 1-3 to be incompatible or resistant, while ratings >3 were compatible or susceptible.

Pathotype designation was executed by adding binary values of the differential genotypes that were compatible with the respective *P. griseola* isolate. For instance, for pathotype 15:39 (virulence phenotype abcdef-ghijkl) in Table 2, the first value was obtained by adding the binary values of the susceptible Andean differential genotype abcdef (1+2+4+8 =15) and the second value was obtained by adding the binary values of the susceptible Mesoamerica genotypes ghijkl (1+2+4+32 = 39) in Table 2 (Pastor-Corrales *et al*., 1998). For consistent results, the experiment was repeated twice.

DNA Extraction and Primer Analysis

The mycelium used in DNA extraction was generated on V8 agar growth media by cutting three disks of 1.5 mm diameter on the edges of actively growing fungi and inoculated on growth media contained in Erlenmeyer flasks (250 ml). The cultures were placed on a rotary shaker (120 rpm) at room temperature for 14 days. Mycelium was harvested through filtration with a cheese-cloth, washed in sterile deionized water, freeze- dried and stored at -24 ^{O}C. DNA was extracted according to Mahuku (2004) and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA). The DNA was adjusted to a standard concentration of 10 ng/ µl before it was used in the PCR reaction. To characterize molecular diversity, four random amplified microsatellites and five conserved sequences (Table 1) were analyzed. The PCR reactions were carried out in 20µl volumes containing 1 × DNA polymerase buffer (100 mM Tris-HCl, 400 mMKCl, 15 mM MgCl2, pH9.0), 3mM MgCl2, 0.4mM dNTPs, 1µM of each primer, 0.3U Taq DNA polymerase (Bioneer Inc. Korea) and 50ng of genomic DNA. A water control (DNA replaced with sterile water) was included with each set of 10 isolates. An additional DNA from isolate RU7 whose PCR amplification product was known was also included as a positive PCR control. The DNA amplification was performed in a Mycycler thermal cycler (Bioneer Inc, Korea) under a program of one cycle at 94 ^{O}C for 5 min, followed by 35 cycles at 94 ^{O}C for 20s, 50 ^{O}C for 40 s and 65 ^{O}C for 8 min and a final 16 min extension at 65 ^{O}C (Table 2). The DNA amplicons were electrophoresed in 1.5% agarose gel for 1 h at 90V in 1X Tris–borate–EDTA buffer (89 mM Tris base, 89mM boric acid–borate and 2mM EDTA pH 8.0), and later stained for 20 min in 0.5 µg/ml ethidium bromide. Gel images were captured using the Gene Snap gel documentation system (SynGene, Frederick MD).

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Table 1: Sequences and annealing temperatures of random amplified microsatellite markers and conserved primers used to amplify P. griseola DNA

Primers	Sequence (5′ to 3′)	Annealing temperature (0°C)	Total number bands	Reference
RAMS 4	AGGGTGTGTGTGTG	40	23	Hantula *et al*., 1996
RAMS 2	TGCCGAGCTG	40	11	"
RAMS 5	GGGTAACGCC	40	11	"
RAMS 6	GTGATCGCAG	40	25	"
BOX A1R	CTACGGCAAGGCGACGCTGACG	50	20	Versalovic *et al*., 1991
ERIC 1R	ATGTAAGCTCCTGGGGAT	50	22	Coenye *et al*., 2002
ERIC 2	AAGTAAGTGACTGGGGGTGAGC	50	22	"
REP1R	IIIICGICGICATCIGGC	40	14	Seurink *et al*., 2003
REP 2	ICGICTTATCIGGCCTAC	40	14	"

Identification of new sources of resistance

Common bean germplasm used in the study was obtained from the Uganda National Bean Programme (UNBP), Namulonge, and the International Centre for Tropical Agriculture (CIAT), Kawanda, in Uganda. A total of 80 bean lines (74 landraces, two checks and four commercial varieties) constituted the germplasm, which was evaluated for *P. griseola* resistance under screenhouse conditions. The landraces that were used had been previously collected from major bean growing areas in Uganda (Okii *et al*., 2014). The resistance and susceptible checks used included: 1) BAT332, which is a domesticated line, small-seeded, and routinely used as one of the differentials for ALS (Mahuku 2002). It is also resistant to race 61:41 (Nietsche *et al*., 2000) and to most Andean and Mesoamerican *P. griseola* races (Buruchara and Buah, 1999), and 2) Kanyebwa, a popular landrace in Uganda, which is susceptible to ALS (Namayanja *et al*., 2006). Commercial varieties included: K131, K132, NABE4 and NABE 13, which are commonly grown in Uganda. Furthermore, four bean lines (U00297, Mexico 54, AND277 and G5686) were also used for the inheritance and allelic tests. U00297 is a small-sized (25g/100 seeds), cream-seeded landrace with a determinate (Type I) growth habit. K131 (MCM5001) and K132 (CAL96) are CIAT-bred lines; K131 and K132 belong to the Mesoamerican and Andean gene pools, respectively. G5686, AND277, and Mexico 54 are resistant to races 31:0, 63:31, 63:39 of *P. griseola* with one to three dominant genes that condition resistance (Carvalho *et al*., 1998; Caixeta *et al*., 2005 and Mahuku *et al*., 2009).

Screening for *P. griseola* resistance

Fungal isolates

Four *P. griseola* pathotypes (1:6, 21:39, 17:39 and 61:63) previously characterized using a set of 12 ALS International bean differential cultivars (CIAT 1995) were sourced from CIAT and used in screening bean lines for resistance. The first three isolates were Mesoamerican types while 61:63 was Andean. These isolates also varied in virulence levels with 1:6 and 61:63 being the least and most virulent, respectively. On the other hand, 21:39 and 17:39 were some of the most prevalent *P. griseola* pathotypes in major bean growing areas in Uganda (Ddamulira *et al*., 2014).

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Screenhouse resistance testing

To identify possible sources of resistance, 80 bean lines were evaluated for ALS resistance under screenhouse conditions. Forest soil was obtained from a nature forest, sand from lake shore and animal manure from an abandoned old cattle kraal with decomposing cow dung. The different soil types were mixed in a ratio of 3:1:1, respectively. Soil samples in three replicates from the mixed soil were analyzed for soil pH, nitrogen (N), phosphorus (P), and potassium (K). The preliminary results indicated soil pH of 6.4, 0.25% N, and 35 and 73 ppm of P and K, respectively. Five seeds of each bean line were sown in 5-litre plastic bucket containing three kilograms of the composite/mixed soil. After attaining three trifoliate leaves, plants were inoculated following procedures described earlier in experiment one. The suspension was applied on the lower and upper surface of leaf using a hand sprayer. The inoculated plants were placed in a humid chamber at approximately 24-27^{O}C with relative humidity of 95 % for 4 days to allow infection to take place as recommended by Hodges and Haasis (1962). The plants were then transferred into the screen house and watered one to two times daily depending on sunshine intensity. The plants were evaluated for ALS resistance according to the Schoonhoven and Pastor-Corrales (1987), as described earlier in determining the pathotypes. The area under disease progress curve (AUDPC) was calculated to determine their reaction type.

Confirmation of resistance

The best way to confirm resistance to fungal pathogens is through repeated screening of materials that prove to be resistant or moderately resistant in the preliminary screening stages. Hence**,** out of 74 landraces, 34 bean lines that were primarily identified to be resistant and moderately resistant to the four pathotypes including four commercial varieties and two checks (BAT 332 and Kanyebwa), were re-screened twice in replicated trials. Similar isolate preparation, inoculation, plant management procedures and disease assessment performed during preliminary evaluation were adhered to in confirmatory evaluation. The area under the disease progress curve (AUDPC) was calculated for each accession using the midpoint rule method (Campell and Madden, 1990) as shown in Equation 1.

$$\text{AUDPC} = \sum_{i=1}^{n=1} [(y_i + y_{i-1})/2](t_{i+1} - t_i) \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (1)$$

where "*t*" is time in days of each evaluation, "*y'* is the disease percentage representing the infected foliage at each evaluation, and n is the number of evaluations.

Inheritance of ALS resistance in U00297

Based on the results from the screening, a study was designed to elucidate the resistance inheritance mechanisms in U00297. A 4 × 4 partial diallel mating design involving four parents (U00297, K132, K131, and Kanyebwa) was executed according to the Griffings (1956) method I, model I, where the parents were crossed in all possible combinations with reciprocals and ignoring self to generate 12 families. U00297 is resistant and the other lines are susceptible to ALS. Different susceptible parents were used to ascertain the nature of resistance genes contained in U00297 in

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different genetic backgrounds. Part of the generated F_1 seed was used to plant a backcrossing program to U00297 (BCR F_1) and to K132, K131, and Kanyebwa (BCS F_1). Another part of the seed was planted to generate F_2 seeds. Thereafter, seeds from the parents, F_1, F_2, and backcross populations were planted for evaluation under screenhouse conditions.

The seeds were sown in 5-litre plastic buckets containing forest soil, lake sand, and animal manure in a ratio of 3:1:1. The experiment was replicated three times and watered regularly to provide moisture. Between 14 and 30 seeds of each parent and F_1 individuals were evaluated depending on seed availability. The number of F_2 individuals evaluated also ranged from 98 to 166 for each cross and 16 to 97 for each backcross population depending on seed availability. Isolate preparation, inoculation, plant management procedures and disease assessment were performed following similar procedures described in the previous experiments.

Allelism of identified resistance source

U00297 was crossed with G5686, AND277, BAT332, and Mexico 54 to generate F_1 and F_2 populations. These four bean genotypes possess complementary resistance genes. The resistance is controlled by one, two, or three dominant genes (Phg_{G5686A}, Phg_{G5686B}, Phg_{5686C}, *Phg-1*, and *Phg-2*) depending on the genotype (Carvalho *et al*., 1998; Caixeta.*et al*., 2005; Namayanja *et al*., 2006, and Mahuku *et al*., 2009). Most of these genes are inherited in monogenic and dominant manner (Caixeta *et al*., 2003; Namayanja *et al*., 2006). In the course of crossing, the crosses involving Mexico 54 and BAT332 failed and only F_1s of U00297 × G5686 and U00297 × AND 277 were obtained. It is probable that Mexico 54 and BAT 332 were not compatible with U00297. Seeds from parents, F_1, F_2 and backcross populations were planted for evaluation under screen house conditions. The seeds were sown in 5-litre plastic pots containing forest soil, lake sand, and animal manure in a ratio of 3:1:1. The experiment was replicated and watered regularly to provide the required moisture for proper growth. Thirty to forty plants of each parent, BC_1F_1 and F_1 individuals were evaluated. The number of F_2 individual plants ranged from 50-157 depending on seed availability. Isolate preparation, inoculation, plant management and disease assessment were performed following similar procedures described in the earlier experiments.

Data analysis

Isolate virulence was determined by considering each differential as a marker and information on virulence phenotype generated by considering incompatible interaction (rating ≤ 3) as absence of a virulence (-) and compatible interactions (rating >3) as presence (+). However, for molecular data, markers were scored as either one (1) for the presence of a band or zero (0) as absence of the band. Only strong and reproducible bands were scored and weak ones discarded. Genetic similarities between all the pairs of isolates were computed using the Jaccard coefficient S (Sneath and Sokal, 1973) in DARwin5. Dissimilarities were computed as genetic distance = 1-S and based on the data, a dendrogram was constructed by an un-weighted pair group method with arithmetic mean (UPGMA) hierarchical clustering using MEGA 5.0. The gene diversity across all loci was estimated (Nei, 1987).

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The area under disease progress curves (AUDPC) was calculated based on disease scores and symptom intensity data to determine the reaction type of each germplasm. Individual plants for each bean line were considered resistant (R) when AUDPC value symptom score ≤ 13.5, intermediate resistant (IR) AUDPC 13.5-27 and susceptible (S) AUDPC > 27. To determine the inheritance pattern, a Chi-squared goodness-of-fit test was performed on data from all crosses in GenStat version 14 (Payne *et al*., 2011). This was done to verify if observed segregation ratios of resistant and susceptible plants fitted the expected Mendelian 3:1 or epistatic 9:7 and 15:1 phenotypic ratios, respectively. Similarly, allelic relationship between resistance sources, segregation ratios for each R × R progeny were computed in GenStat and genetic hypotheses tested for significance for each population using the chi-squared goodness-of-fit test to determine the deviation of observed frequencies from the hypothesized ratios.

Results

Virulence

The reaction of 45 *P. griseola* isolates on differential bean cultivars revealed the existence of pathogenic variability in this fungus. In total, 12 pathotypes were defined out of the 45 isolates studied (Table 2). Most isolates infected Andean and Mesoamerica bean differentials with only two isolates that were more infectious to Andean than Mesoamerica differentials (Table 2). Based on isolates' reactions on the differential bean cultivars, 43 isolates were classified as Middle American and two as Andean groups, respectively. Pathotype 61:63 was compatible with eleven differential cultivars tested, while 17:23 and 21:39 were the most common isolates in the study (Table 2).

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Table 2: Response of bean differential cultivars to inoculation with Ugandan *Pseudocercospora griseola* isolates

Andean group[a]						Mesoamerican group[b]						Pathotype[c]	Number of isolates
a	b	c	d	e	f	g	h	I	j	k	l		
1	2	4	8	16	32	1	2	4	8	16	32		
+	-	+	-	+	-	+	+	+	-	-	+	21:39	8
+	-	-	-	+	-	+	+	+	-	-	+	17:39	5
+	-	-	+	-	+	-	+	+	-	-	-	41:6	2
+	-	+	-	-	-	+	+	+	+	+	-	5:31	2
+	-	+	-	+	-	+	+	+	-	-	+	21:38	4
+	-	+	-	-	-	+	+	+	-	+	+	5:55	2
+	-	+	-	-	-	+	-	+	+	-	-	13:13	2
+	-	+	-	-	-	-	+	+	+	+	-	5:30	4
+	-	+	-	-	-	-	+	+	-	-	-	5:6	2
+	-	-	-	+	-	+	+	+	-	+	-	17:23	11
+	-	+	+	-	-	+	+	+	-	+	+	13:55	2
+	-	+	+	+	+	+	+	+	+	+	+	61:63	1

[a,b]Andean groups included cultivars: (a) Don Timoteo; (b) G 11796; (c) Bloom Bayo; (d) Montcalm; (e) Amendoin; (f) G 5686. Middle American group included cultivars: (g) Pan 72; (h) G 2858; (i) Flor de Mayo; (j) Mexico 54; (k) BAT 332; (l) Cornell 49–242. [c]Pathotype designation is based on the sum (binary values) of bean cultivars with 10 scale value. (+), Compatible reaction; (-), Incompatible reaction. All pathogenicity tests included three replicates per isolate

Molecular Characterisation of *P. griseola*

Based on the nine primer pairs used to analyze the pathogenic variability of *P. griseola,* the 45 isolates were grouped into two main clusters, with an average dissimilarity of 0.98. The Middle American and Andean groups constituted 25 and 20 isolates with one control for each group, respectively. The two main groups were clustered into sub-groups; the Andean group consisted of two sub-groups with average dissimilarity of 50%, while the Middle America was divided into three sub-groups with 98% dissimilarity. The combination of markers used in the study identified 30 haplotypes. Analysis of molecular variance (AMOVA) indicated that most of the variation resulted from genetic difference within the Middle American and Andean groups (68.4%, $P<$ 0.05) rather than among the main groups (31.6%). The analysis of genetic diversity indicated that the pathogen was highly variable with the genetic diversity of the entire population being 0.901 and that of Middle American and Andean gene pools being 0.450 (SD 0.098) and 0.443 (SD 0.061), respectively.

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Screening for *P. griseola* resistance

In a first screening, AUDPC ranged from 30.2-40.5 among 74 landraces. The analysis of variance for AUDPC among these landraces indicated that there were significant AUDPC differences ($P < 0.05$) among landraces for the four *P. griseola* pathotypes (1:6, 17:39, 21:39 and 61:63) (Table 3). Out of 74 landraces screened, 14% were rated as resistant (< 13.5) with no symptoms observed on the leaves, 22% were moderately resistant (13.5- 27.0) with small lesions on leaves and limited sporulation, while 54% were considered to be susceptible (>27.0) to *P griseola*.

Table 3: Analysis of angular leaf spot severity on 74 landraces and 4 commercial bean varieties under screenhouse conditions at Kawanda based on four *Pseudocercospora griseola* isolates in 2011

Pathotype	Landraces					Commercial varieties				
	DF	MS	AUDPC	SED	CV	DF	MS	AUDPC	SED	CV
1:6	3	688.8**	30.2	3.39	3.8	3	689.1**	28.4	2.90	12.5
17:39	3	381.6**	32.7	8.29	1.1	3	111.1**	42.2	2.34	20.1
21:39	3	334.1**	34.5	5.53	19.6	3	NS	29.3	9.70	4.5
61:63	3	169.6**	40.5	6.45	9.5	3	NS	28.1	6.20	25.0

** P<0.01, NS- not significant P >0.05, CV-coefficient of variation in percentages, MS- Mean square of ALS severity leaves, DF- Degrees of freedom, SED-standard error of difference, AUDPC-area under disease progressive curve

On the other hand, significant ($P<0.05$) differences for AUDPC among commercial varieties were observed only for two pathotypes, 1:6 and 17:39 (Table 3). The reaction of 34 landraces (which were resistant or moderately resistant to four pathotypes in the first screening trial) to inoculation of individual pathotypes was significantly ($P<0.05$) different (Table 3). The AUDPC values for pathotypes 1:6, 21:39, 17:39 and 61:63 ranged from 4.5-40.5, 9-32.8, 5.8-36.9 and 12.9-35.2, respectively. Most landraces (62.5%) were resistant (< 13.5) to pathotype 1:6; in contrast, a majority was susceptible (70%) to pathotype 61:63 (Table 4), which is among the most virulent pathotype in Uganda. Forty-seven percent of the screened bean lines were moderately resistant (rating 13.5 - 27.0) to pathotype 21:39, while the smallest percentage (17.5%) of screened bean lines was moderately resistant to 1:6. Apart from the resistant check (BAT332), only landrace U00297 was resistant to all the four pathotypes. Three landraces (U0074, U351 and U1-9) were resistant to three pathotypes but susceptible to the most virulent pathotype 61:63.

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Table 4: Reaction of 40 common bean lines to inoculation with four *Pseudocercospora griseola* pathotypes under screen house conditions at Kawanda in 2010

	ALS REACTION							
	1:6		21:39		17:39		61:63	
Bean lines	**AUDPC**	**RC**	**AUDPC**	**RC**	**AUDPC**	**RC**	**AUDPC**	**RC**
Landraces								
U0041	9.0	R	22.5	I	14.0	I	27.6	S
U0074	4.5	R	9.0	R	9.0	R	34.2	S
U351	9.0	R	13.5	R	13.5	R	28.3	S
U0066	9.0	R	13.5	R	31.5	S	28.7	S
U1-9	7.2	R	13.5	R	10.8	R	33.1	S
U0077	9.0	R	18.0	I	18.9	I	33.2	S
U614	13.5	R	14.8	I	21.6	I	31.6	S
U620	9.0	R	25.2	I	14.4	I	29.2	S
U0082	14.8	I	13.5	R	9.0	R	22.3	I
U204	19.3	I	25.2	I	31.5	S	32.3	S
U00335	9.0	R	13.5	R	15.5	I	28.4	S
U0043	11.7	R	19.5	I	8.55	R	18.9	I
U284	5.8	R	20.7	I	8.55	R	17.4	I
U608	10.4	R	20.7	I	30.1	S	30.8	S
Masindi yellow	7.2	R	32.8	S	14.4	R	23.1	I
U650	38.7	S	16.2	I	36.9	S	32.4	S
U342	13.5	R	23.8	I	27.5	S	24.4	I
U00297	9.0	R	13.5	R	7.6	R	12.9	R
U00101	5.9	R	31.5	S	19.8	I	32.4	S
U274	14.8	I	23.8	I	24.7	I	28.9	S
U0049	14.4	I	14.9	I	12.2	R	14.7	I
U0068	36	S	30.1	S	20.3	I	32.2	S
U0070	9.0	R	37.3	S	18.0	I	35.2	S
U0080	11.7	R	27.0	I	24.3	I	33.2	S
U0083	13.5	R	28.3	S	29.7	S	32.7	S
U0085	16.2	I	30.1	S	5.82	R	20.8	I
U00212	9.0	R	16.2	R	16.2	R	15.7	I
U609	13.5	R	22.5	I	27.0	I	27.8	S
U653	19.4	I	34.2	S	24.7	I	29.1	S
U659	40.5	S	31.5	S	27.5	S	33.3	S
U0010	40.5	S	14.8	I	29.3	S	34.8	S
U635	11.7	R	19.3	I	17.6	I	29.1	S
U0053	4.5	R	18.0	I	17.1	I	24.4	I
U1-5	25.2	I	23.8	I	36.4	S	32.4	S
Checks								
BAT332	4.5	R	4.5	R	5.5	R	4.0	R
Kanyebwa	27.4	S	30.2	S	37.8	S	32.2	S
Commercial varieties								
K131	37.3	S	27.4	S	28.5	S	32.7	S
K132	28.4	S	29.7	S	28.8	S	33.2	S
NABE13	10.8	R	16.7	I	12.6	R	18.4	I
NABE4	28	S	31.2	S	32.9	S	33.7	S
Mean	15.7		21.8		20.5		27.5	
LSD ($_{0.05}$)	6.3		12.1		15.3		16.0	
CV%	26.4		37.6		34.8		46.2	

AUDPC=Area under disease progress curves RC=Resistance conditions, R=Resistant, S=Susceptible, I=intermediate resistance

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Inheritance of resistance to *P. griseola*

U00297 was resistant (AUDPC < 13.5) to pathotypes 17:39, 21:39 and 61:63, while parents K131, K132 and Kanyebwa were all susceptible (AUDPC >13.5) to the same pathotypes. Pathotype 1:6 was excluded from those used for inheritance study due to loss of viability that led to no observable disease symptoms appearing on plants inoculated with it. Most F_1 plants grew healthy with no visible diseases' symptoms', suggesting that ALS resistance was possibly inherited in a dominant manner. The chi-squared test indicated that segregation of ALS resistance in F_2 population of crosses KB × U00297 and K131 × U00297 when inoculated with 61:63 and 17:39 fitted the tested ratio 9:7 (Table 5). The best fit to 9:7 in these crosses suggests that they segregated for at least two genes. In contrast, crosses K132 × U00297 and K131 × U00297 when inoculated with 61:63 and 21:39, respectively, exhibited a segregation ratio of 7:9, suggesting the presence of complementary epistatic gene interactions (Table 5). F_2 populations K132 × U00297 and KB × U00297 fitted the test ratio of 3:1 when inoculated with 17:39. However, cross KB × U00297 failed to fit the same test ratio when it was inoculated with 21:39 (Table 5). The segregation ratios in the backcross populations fitted the expected segregation ratios 1:1 and 1:0, respectively, except for the backcross with resistant parent (U00297) when it was inoculated with 17:39 (Table 5).

Table 5: Reaction of F_2, back cross progenies to inoculation of three P. *griseola* pathotypes under screen house conditions at Kawanda

Populations	Pathotypes	Total no. of plants	Observed plants		Expected ratio		
			R	S	R:S	x^2	P-value
F_2 (K131 x U00297)	61:63	157	72	85	7:9	0.2839	0.5940
F_2 (K132 x U00297)	61:63	166	74	92	7:9	0.0462	0.8296
F_2 (KB x U00297)	61:63	77	47	30	9:7	0.7176	0.3969
BC_{K132}	61:63	58	27	31	1:1	0.2759	0.5994
BC_{K131}	61:63	53	25	28	1:1	0.1698	0.6803
BC_{KB}	61:63	61	32	29	1:1	0.8251	0.3637
BC_{U00297}	61:63	47	45	2	1:0	0.0957	0.1915
F_2 (K131 x U00297)	17:39	98	62	36	9:7	1.9598	0.1615
F_2 (K132 x U00297)	17:39	98	70	28	3:1	0.6663	0.4142
F_2 (KB x U00297)	17:39	157	123	34	3:1	0.9363	0.3332
BC_{K132}	17:39	59	28	31	1:1	0.0763	0.1525
BC_{K131}	17:39	67	35	32	1:1	0.1343	0.7140
BC_{KB}	17:39	54	29	25	1:1	0.2963	0.5862
BC_{U00297}	17:39	97	95	2	1:0	0.0000	0.0412
F_2 (K131 x U00297)	21:39	102	41	61	7:9	0.5234	0.4693
F_2 (KB x U00297)	21:39	111	72	39	3:1	6.0810	0.0136
BC_{K132}	21:39	47	25	22	1:1	0.0000	0.0851
BC_{K131}	21:39	64	30	34	1:1	0.2500	0.6171
BC_{KB}	21:39	81	42	39	1:1	0.1111	0.7389
BC_{U00297}	21: 39	16	16	0	1:0	0.0000	1.0000

R: resistant, S: susceptible, Chi-square P- values greater than 0.05 indicate that the observed values were not significantly different from the expected value

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Testing allelic relationship between resistance genotypes

The allelic relationship between angular leaf spot resistance gene in landrace U00297 and other resistance genes previously characterized in cultivars G5686, AND277 and Mexico 54 are presented in Table 6. The segregation of ALS resistance in the allelism test fitted 15 resistant: 1 susceptible and 63 resistant: 1 susceptible ratio, which exhibited the action of dominant genes conferring resistance to 17:39, 21:39 and 61:63. The chi-square x^2 values showed a good fit for a segregation ratio of 15 resistant to 1 susceptible in three F_2 populations from crosses U00297 × G5686, U00297 × AND 277 and G5686 × AND 277 (Table 5), which demonstrates the presence of two dominant genes that confer resistance to pathotypes 17:39 and 21:39 of *P. griseola.*

Table 6: Reaction of F_2 progenies derived from resistant parents to inoculation of 61:63, 17:39 and 21:39 *Pseudocercospora griseola* pathotypes under screen house conditions at Kawanda

F_2 populations	Pathotypes	Total No. plants	Observed plants		Expected ratio	x^2	P-value
			R	S	R:S		
G5686 x U00297	61:63	104	102	2	63:1	0.1049	0.7460
AND 277 x U00297	61:63	111	105	6	15:1	0.0567	0.8119
AND 277 x Mexico 54	61:63	85	85	0	15:1	5.6667	0.0173
AND 277 x G5686	61:63	103	97	6	15: 1	0.0026	0.9791
G5686 x U00297	17:39	100	92	8	15:1	0.9131	0.3393
AND 277 x U00297	17:39	94	88	6	15:1	0.0456	0.8308
AND 277 x Mexico 54	17:39	97	97	0	15:1	6.4667	0.0110
AND 277 x G5686	17:39	98	92	6	15:1	0.0110	0.9163
G5686 x U00297	21:39	56	51	5	15:1	1.0735	0.3002
AND 277 x U00297	21:39	108	101	7	15:1	0.0735	0.7835
AND 277 x Mexico 54	21:39	96	96	0	15:1	6.4000	0.0114
AND 277 x G5686	21:39	105	98	7	15:1	0.1269	0.7216

R: resistant, S: susceptible, chi-square P values greater than 0.05 indicate that the observed values were not significantly different from the expected value

These results support the hypothesis that the gene conferring resistance to pathotypes 17:39 and 21:39 of this fungal pathogen, present in U00297, is independent from other genes (*Phg-1*, *PhgG5686A*), harboured in AND 277 and G5686, respectively. In addition, the allelism test applied to the cross AND277 × G5686 had a segregation ratio of 63R:1S, which exhibited the action of three dominant genes that confer resistance to pathotype 61:39. This also indicated independence of AND277 genes from *PhgG5686A*, *PhgG5686B* and *PhgG5686C* genes. No susceptible plants were observed in the population from the cross Mexico 54 × AND277, which indicated that the resistance gene in the two cultivars co-segregate and are either in same locus or are closely linked genes. On the other hand, all the G5686 × Mexico 54 crossed flowers aborted probably due to incompatibility as described by Shii *et al.* (1980) and Gepts and Bliss (1985).

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Discussion

Most fungal pathogens that affect common beans are highly variable (Mahuku *et al*., 2009). This study also demonstrated high variability in *P. griseola.* Based on the pathogenicity reactions of *P. griseola* on standard bean differential cultivars, the pathogen was divided into Middle American and Andean strains, corresponding to two gene pools of common bean. These results are consistent with Guzmán *et al*. (1995) and Pedro *et al*. (2006) who reported that the causal organism for ALS underwent a micro-evolution with its host, leading to two distinct groups, the Middle American and Andean groups. This was also evidenced in the study when the reactions of isolates to differentials of known Andean and Middle American origin were compared. Andeans isolates were more virulent to large-seeded (Andean) cultivars, hence categorizing *P. griseola* isolates from Uganda into two major groups.

Among the two groups defined by differentials, the majority of the isolates belonged to Middle American group, yet in Uganda beans of Andean origin are the predominant bean type. This indicated that some of the isolates that where recovered from Andean genotypes might not have been necessarily Andean type. This was possible because Middle America isolates are also known to attack bean genotypes from the Andean gene pool (Araya *et al*., 2004). In addition, previous studies by Guzmán *et al*. (1995) in Malawi indicated the possibility of finding Middle American isolates on Andean host genotypes. This implied that in Uganda the existing *P. griseola* race may not be dependent on the bean type predominantly grown.

The study identified pathotype 61:63 among Ugandan isolates, which was compatible with eleven differential cultivars. Pathotype 61:63 overcomes resistant genes in 11 known sources of resistance that constituted part of the differential set. Similar pathotypes have been reported in Brazil and Argentina (Sartorato, 2002; Sebastian *et al*., 2006) and their presence in Uganda and other countries implies that new sources of resistance need to be identified regularly to mitigate resistance break down. On the other hand, pathotypes that overcome resistance in differential cultivars are potential candidate for use in screening germplasm against ALS by breeding programs.

The common occurrence of pathotypes 17:23 and 21:39, exhibited in the study indicated an overlap of pathotypes across bean production areas. Such pathotype overlap could possibly be a result of seed exchange, which is a common practice among smallholder farmers in Uganda (David *et al*., 2000). Due to high cost of certified seed, farmers source seed from fellow farmers or informal markets whose supplies come from different parts of the country. Such a seed system is likely to transmit different *P. griseola* races across bean production areas. It was also worth noting that the frequently occurring pathotypes 17:23 and 17:39 were being reported for the first time in Uganda. This could have been attributed to previous studies, which were continental or regional, without comprehensive countrywide studies covering all bean production areas across the country. For instance, among the 7 and 16 Ugandan isolates studied by Aggarwal *et al*. (2004) and Mahuku *et al*. (2002), respectively, under a regional study in eastern and southern Africa, none of the isolates were in the same pathogenic class like 17:23 and 17:39 identified in our study.

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In this study, 45 isolates were studied, among which a molecular analysis identified 30 haplotypes. This meant that with more extensive isolate collection covering the whole country, more pathogen variability could be revealed. The genetic diversity of *P. griseola* fungus was studied based on molecular markers. At the DNA level, high diversity was exhibited with markers defining 30 haplotypes compared to virulence which defined 12 pathotype. This was expected because molecular markers are unrelated to pathotype diversity (Sebastian *et al*., 2006) suggesting that isolates of the same pathotypes might not necessarily be closely related based on DNA analysis. These results are consistent with findings by Sicard *et al*. (1993) and Liebenberg and Pretorius (1997), who provided evidence that there is high haplotypic diversity in *P. griseola*. The high genetic differentiation among the Andean and Middle American groups revealed a strong influence of host specialisation on the population structure of *P. griseola*. It also confirmed that the sub-groups of this pathogen are highly variable and structured along host gene pools (Guzmán *et al*. 1995; Pastor-Corrales *et al*., 1998). Genetic differentiation within each group was high (68.4%), indicating that high genetic diversity is maintained in the fungus. However, the sources of genetic diversity observed among isolates were uncertain for a fungus like *P. griseola* with no sexual cycle reported (Liebenberg and Pretorius, 1997). However, factors such as mutations, migration and the parasexual cycle (Anderson and Kohn, 1995; Brown and Wolfe, 1990; McDonald and McDermott, 1993; Zeigler *et al*., 1995) can interact to create or maintain high levels of genetic diversity as observed in *P. griseola*. Zeigler *et al*. (1995) showed evidence that high levels of haplotypic diversity can be maintained in asexually reproducing fungi through parasexual reproduction. Chromosomal diversions, deletions and loss of chromosome segments (Kristler and Miao, 1992) and the presence of transposons (Kempken and Kuck, 1998) all have the capacity to increase the diversity in fungi and contribute to high haplotypic diversity (Liebenberg and Pretorius, 1997). Seed transmission of *P. griseola* has been adequately documented (Sartorato, 2000), and introductions of new haplotypes through contaminated seed cannot be ruled out as a source of high diversity (Leibenberg and Pretorius, 1997). Since this study was unable to identify the actual cause of high differentiation, we suggest that further research is undertaken to validate whether the observed population differentiation contributed to the factors mentioned above.

Several studies have reported high genetic variability of *P. griseola* defined by different markers. For instance, studies by Sartorato (2004) estimated high genetic variability of 96 *P. griseola* using RAPD markers though the grouping was not based on geographical origin. Other studies on estimation of genetic variability have been conducted by Sebastian and Balatti (2006) in Argentina; out of 45 *P. griseola* isolates, a combination of ISSR and RAPD defined 18 haplotypes. Similarly, in Brazil, Abadio *et al*. (2012) defined 27 haplotypes out of the 27 isolates using ISSR markers, while in our study a combination of RAMS and REP markers has revealed 30 haplotypes indicating high variability among *P. griseola* isolates existing in Uganda.

Developing resistant bean cultivars partly depends on variability expressed by the disease-causing pathogen. Since most fungal diseases are spread by highly variable pathogens, it is important to continuously diversify sources of resistance as a strategy to

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control angular leaf spot and rationalize the breeding process. We identified landrace U00297 to be resistant to four *P. griseola* pathotypes 1:6, 17:39, 21:39 and 61:63 under screen house conditions. In some genetic backgrounds, resistance in U00297 is conferred by a single dominant gene, which is independent of resistance genes found in cultivars AND277 and G5686, while in others, resistance is due to epistatic gene interaction involving two or three genes. Resistance in U00297 has been successfully transmitted into certain F_2 progenies. This is evident in our study by F_2 plants which were resistant to *P. griseola* pathotype 17:39.

The screening process revealed variation in reaction of bean lines to Ugandan *P. griseola* pathotypes. Only U00297 was resistant to four pathotypes, indicating low levels of resistance among the other bean lines evaluated and the complexity of managing *P. griseola* in bean-growing areas. Nonetheless, the resistant line identified could be a good source of resistance, which can supplement other existing resistance sources to develop durable ALS resistance. Given the fact that U00297 is resistant to pathotypes, 17:39 and 61:63, which are among the most prevalent and virulent pathotypes in Uganda, it constitutes a resistant source that can provide desired resistance to commercial bean varieties in Uganda that are known to be susceptible to ALS (Opio *et al.,* 2001). Our findings were in line with earlier studies by Mahuku *et al*. (2002), which also identified four bean accessions in a core bean collection that were resistant to pathotype 63-63 (one of the most virulent pathotypes that overcomes resistance in differentials) under screen house conditions. Similarly, Wagara *et al*. (2007) identified 13 bean genotypes that were resistant to at least 40 *P. griseola* pathotypes in Kenya. Similarly, U00297 has a potential of being used to improve resistance against ALS among susceptible commercial bean varieties in Uganda.

One approach to ensure continued improvement of ALS resistance in bean varieties is through understanding the inheritance and segregation pattern in new sources of resistance. This is pertinent in breeding because it offers breeders an opportunity to design strategies that maximizes efficiency in developing improved resistant cultivars. Our findings showed that F_1 plants were resistant to most pathotypes, suggesting that resistance in U00297 is inherited as a dominant trait. The monogenic inheritance of resistance indicates that pedigree or backcross breeding would be adequate to transfer resistance to susceptible lines. Similarly, segregation for resistance in F_2 K132 × U00297 and KB × U00297 populations was consistent with a ratio of 3:1 as resistant: susceptible, which further confirmed that U00297 resistance to pathotype 17:39 was due to a single dominant gene.

The dominant nature of resistance in U00297 cultivar revealed that resistance transfer into KB and K132 is possible through conventional breeding, provided that both alleles transferred are dominant alleles. Muthoni *et al*. (2011) and Caixeta *et al*. (2003) reported similar resistance inheritance patterns in other ALS resistant sources. Similarly, inheritance to *P. griseola* in Mexico 54 and BAT332 is also reported to be monogenic with a single dominant gene effect. Mahuku *et al*. (2009) also reported that ALS resistance in bean cultivar G5686 to pathotypes 31-0 was conditioned by a single or three dominant genes. However, previous inheritance studies have revealed that

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resistance to *P. griseola* is conditioned by few genes that can either be recessive or dominant depending on the cultivar used as a susceptible parent (Carvalho *et al.*, 1998).

It was further observed that segregation ratios in F_2 population deviated from the expected ratios, indicating that resistance of U00297 to pathotypes 21:39 and 61:39 involved digenic epistatic gene interactions. In this study, G5686 × U00297 (R × R) yielded a ratio of 63R:1S in the F_2 generation, when inoculated with pathotypes 61:63, suggesting segregation of three unlinked resistance genes. Because Mahuku *et al.* (2009) posited the existence of three resistance genes in G5686 and our current, one of them was shared between G5686 and U00297.

Conclusion

The variation in *P. griseola* was revealed by the existence of 12 pathotypes and 30 haplotypes, which were defined by standard bean differential cultivars and molecular makers. This exhibited the potential of standard differentials and molecular markers to detect isolate polymorphism though molecular markers proved to detect more variation than differentials can. On the other hand, the variation exhibited by this pathogen indicates that different sources of resistance will be required to manage ALS; hence, concerted efforts are needed to develop broad resistance for this disease in Uganda. On the other hand, the study identified landrace U00297 as a potential source of resistance to four *P. griseola* pathotypes. Resistance to pathotype 17:39 in U00297 is inherited in a dominant manner. It is, therefore, possible to adequately transfer resistance in U00297 into K132 and Kanyebwa using pedigree breeding. Resistance gene that confers resistance in U00297 is independent of resistance genes harboured by AND-277 and G5686. This information will aid breeding programs targeting improving resistance to ALS using U00297 as the parent.

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