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Morphological Characterization of Ugandan Isolates of *Sphaceloma* sp. Causing Cowpea Scab Disease

Emmanuel Afutu[1,2], Eric E. Agoyi[1], Fred Kato[3], Robert Amayo[4], Moses Biruma[4] & Patrick R. Rubaihayo[1]

[1] Department of Agricultural Production, School of Agriculture, College of Agricultural and Environmental Sciences, Makerere University, Kampala, Uganda

[2] Department of Crop Science, School of Agriculture, College of Agriculture and Natural Sciences, University of Cape Coast, Cape Coast, Ghana

[3] International Centre for Tropical Agriculture (CIAT), Kampala, Uganda

[4] National Semi-Arid Resources Research Institute (NaSARRI), Serere, Uganda

Correspondence: Emmanuel Afutu, Department of Agricultural Production, School of Agriculture, College of Agricultural and Environmental Sciences, Makerere University, P. O. Box 7062, Kampala, Uganda. E-mail: mmanuelaf@yahoo.com

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Abstract

Scab is an important fungal disease of cowpea, affecting both young and old tissues including stems, leaves and pods of susceptible cowpea genotypes, leading to significant yield losses of up to 100% under severe infections. Colony characteristics on agar media, symptomatology, phylogenetic affinity of hosts and host range have been used to justify taxonomic distinctions. The correct identification and description of a pathogen is paramount in understanding its control or developing genotypes resistant to it. This study involved the isolation and culture of the scab fungus (*Sphaceloma* sp.) from infected plant parts (leaves and pods) collected from farmers' fields across major cowpea growing districts and agro-ecological zones in Uganda. The fungus was characterized using growth habit on potato dextrose agar (PDA) media, conidia features, variability in radial growth rate (mm/day) among the isolates and pathogenicity and virulence of some isolates on 20 selected cowpea genotypes with different levels of resistance. A total of 495 *Sphaceloma* sp. isolates comprising of 419 from infected leaves and 76 from infected pods were obtained following isolation and culture. There was a wide variation in the isolates based on the amount, nature, colour, depth and rate of mycelia growth, features of conidia and number of septations. Based on the mean incidence, severity, AUDPC and pathogenicity on the 20 genotypes, the isolates were put into three pathogenicity groups. Isolates were mostly slow growing (> 14 days to cover entire 90 mm petri dish). Genotypes NE 31 and NE 70 showed broad spectrum of resistance to the isolates and could therefore be recommended as parental lines in the cowpea breeding programme to develop cultivars with wide horizontal resistance to the scab disease.

Keywords: pathogenicity, virulence, horizontal resistance, radial growth rate, variability

1. Introduction

Cowpea scab, caused by the fungus *Sphaceloma* sp., is one of the major diseases affecting the production of cowpea in Uganda. The disease is capable of causing up to 100% yield loss (Mbong et al., 2010; 2012). Scab disease is widespread in all the cowpea growing districts of Uganda with mean incidence ranging between 35-70% and mean severity 2-4 out of a severity scale of 1-5, where 1 = no symptoms, and 5 = more than 50% infection, based on which Amuria and Tororo districts were reported to be hot spots of the disease in the country and was found to be more severe at higher altitudes (> 1200 m.a.s.l.) (Afutu et al., 2016a).

The genus *Sphaceloma* is made up of more than 50 species, of which most are found in the Tropical and Sub-tropical regions and have been characterized based on the colony characters on agar media, symptomatology, phylogenetic affinity of reported hosts, and to a lesser extent, host range (Zeigler & Lozano, 1983). The fungus is the imperfect state of *Elsinoe* and is considered as lesser fungi because it lacks the perfect stage (sexual reproduction). Though imperfect, it is part of important eukaryotic microorganisms which affects plants and other life forms in diverse ways and thus, the need to determine its identity is important in research, industry,

plant pathology and many other disciplines (Barnett & Hunter, 1987). The genus *Sphaceloma* has been described by Barnett and Hunter (1987) as having disc-shaped or cushion-shaped acervuli, waxy; conidiophores simple, closely grouped or compacted, arising from a stroma-like base, sometimes almost appearing as a sporodochium; conidia hyaline, 1-celled, ovoid or oblong; parasitic; imperfect states of *Elsinoe*; similar to *Gloeosporium* and *Colletotrichum*. On the other hand, Ayodele and Kumar (2014) described the genus as having hyaline mycelium, scanty and submerged; hyaline to pale coloured conidia which are produced in pycnidia; have hyaline ascospores borne on the asci; pale coloured oblong to elliptical, and 3 septate. The conidia of isolates of the genus *Sphaceloma* were described as being small, unicellular, and hyaline, formed in more or less acervulus-like structures or, more commonly on continuous fertile layers of densely packed phialidic conidiophores with some species forming a larger, 0-2 septate, pigmented, thick-walled, spindle-shaped spores under certain conditions (Zeigler & Lozano, 1983). The conidia of the genus were later described as small, thin-walled, ellipsoid to (rarely) globose, commonly with one or two guttules and conidiophores being phialides hyaline to slightly pigmented 0-1 septate while conidiophores from the weedy species were phialides, hyaline brown 0-2 septate producing hyaline conidia (Alvarez et al., 2003).

The fungus attacks all the above ground parts of the cowpea plant (Emechebe, 1980; Iceduna, 1993; Mbong et al., 2010, 2012). Infected leaves show spots on both leaf surfaces, cupped, appearance of small grayish lesions along the veins leading to leaf distortions and ragged appearance under severe infections while infected stems show oval to elongated silver grey lesions surrounded by red or brown elliptical rings with lesions coalescing and forming distortions under severe infections. On the other hand, sunken spots with grey centres surrounded by brown borders appear on infected pods, leading to malformation and formation of dark coloured pycnidia in the brown spots, with heavily scabbed young pods aborting or remaining attached to the plant as mummified black masses (Singh et al., 1997).

Screening of local germplasm for sources of resistance to the scab disease in Uganda showed a wide variability in response to the disease where lines which were rated resistant in one location, were found to be either moderately resistant or susceptible in another location (Afutu et al., 2016b). The variability in response to the disease, among other factors, suggested a variability in pathotypes of the scab fungus. This phenomenon complicates crop protection programs (Alvarez et al., 2003) and the development of resistant cultivars. This study was therefore, conducted to determine the morphological variability, growth rate and pathogenicity of some selected isolates on some local germplasm having varying levels of resistance to the scab disease to identify cowpea lines with broad spectrum of resistance for possible parents in the cowpea breeding programme.

2. Materials and Methods

2.1 Sampling, Isolation, Culture and Morphological Characterization of Pathogen

Samples of infected plant materials were collected in a field survey conducted in some of the major cowpea growing areas in the country, for isolation and culture of the scab fungus. Infected leaves and pods were cut into small portions using sterile surgical blades and were disinfested with 75% ethanol for 1 minute and then in 1% sodium hypochlorite for 1 minute, followed by rinsing in sterile water (Hou et al., 2014). The intact lesions were plated on commercial preparations of Potato Dextrose Agar (PDA) (Difco, Detroit) amended with Streptomycin Sulphate (1.5 g/L) and rose bengal (0.0025 g/L of agar) for isolation of *Sphaceloma* sp. (Mungo et al., 1998). Five lesions from each cowpea plant part were plated on the media in 9 cm-diameter Petri dishes and incubated at 26 ^{o}C for 5-7 days.

Following isolation and culture, a total of 495 pure fungal isolates from single conidium cultures were morphologically characterized based on colony characters such as the texture, density, colour, presence of conidial masses and colour of the reverse side of the dish (Talhinhas et al., 2002). The isolates were obtained from a total of 14 districts with each having three Sub-Counties and three farms per Sub-County making 126 farms in total. Isolates were named by assigning unique two-letter codes to represent the districts of origin and followed by serial numbers to identify the particular isolates (Table 1) and a letter “L” or “P” to indicate that the isolate was obtained from an infected leaf or pod respectively.

2.2 Pathogenicity of Selected Isolates

Five isolates were selected for pathogenicity tests based on the Agro-ecological zone and Districts where the crop was mostly cultivated. The number of isolates chosen from a region was based on the size of the region covered during the field survey and the morphological groups to which the isolate belonged. Pathogenicity of the five selected isolates was determined by inoculating seedlings of 20 cowpea genotypes (Table 2) selected on the basis of their resistance ratings following field screening under natural infections (Afutu et al., 2016b). Seeds of each of the 20 lines were sown in 20 cm diameter by 20 cm high plastic buckets filled with sterilized top soil

with one seed per bucket. The plants were grown in the screen house and no supplementary lighting was provided. Temperature ranged from 19 ^{o}C at night to 27 ^{o}C during the day. Supplementary irrigation was applied where necessary by directly watering and avoiding water coming into contact with the surface of the leaves. Relative humidity was mainly between 50-80%.

Table 1. Names of Districts and Sub-counties from which isolates (infected plant parts) were obtained

Agro-Ecological Zone	District	Sub-County	Given Code	Isolates
Eastern Agro-Ecological Zone	Soroti	Soroti	ST	1-12
	Soroti	Gweri	ST	13-24
	Soroti	Arapai	ST	25-36
	Serere	Kyere	SE	37-48
	Serere	Atira	SE	49-60
	Serere	Kateta	SE	61-72
	Kumi	Kumi	KU	73-84
	Kumi	Atutu	KU	85-96
	Kumi	Bukedea	KU	97-108
	Palisa	Kameki	PA	109-120
	Palisa	kamuge	PA	121-132
	Palisa	Opwateta	PA	133-144
	Tororo	Kisoko	TR	145-156
	Tororo	Mollo	TR	157-168
	Tororo	Mella	TR	169-180
	Amuria	Kuju	AM	181-192
	Amuria	Asamuk	AM	193-204
	Amuria	Orungo	AM	205-216
North Eastern Savannah Grassland	Pader	Pajule	PD	217-228
	Pader	Puranga	PD	229-240
	Pader	Kilak	PD	241-252
	Kitgum	Kitgum	KT	253-264
	Kitgum	Matidi	KT	265-276
	Kitgum	Amida	KT	277-288
	Apac	Akalo	AP	289-300
	Apac	Aduku	AP	301-312
	Apac	Abomola	AP	313-324
	Dokolo	Dokolo	DK	325-336
	Dokolo	Agwata	DK	337-348
	Dokolo	Adok	DK	349-360
	Lira	Lira	LR	361-372
	Lira	Agweng	LR	373-384
	Lira	Ngetta	LR	385-396
North Western Savannah Grassland	Nebbi	Nebbi	NB	397-408
	Nebbi	Pakwach	NB	409-420
	Nebbi	Kucwiny	NB	421-432
	Arua	Arua	AR	433-444
	Arua	Oluko	AR	445-456
	Arua	Manibe	AR	457-468
	Yumbe	Yumbe	YU	469-480
	Yumbe	Odravu	YU	481-492
	Yumbe	Kululu	YU	493-504

Inoculum was prepared by culturing isolates in half strength (18 g/L) Potato Dextrose Broth (PDB) (HiMedia Laboratories Pvt., India) supplemented with Rifampicin antibiotic at 0.03 g/L, Streptomycin Sulphate at 1.5g/L and Rose Bengal at 0.0025 g/L. Glass wares containing the media and inoculum were put on a magnetic shaker (Stuart Scientific Flask Shaker SF1) set to 100 oscillations per minute (osc/min) for 10 days to prevent mycelia formation within the culture and to promote conidia formation (Waller, 2002). The concentration of inoculum was determined using Neubauer improved bright-light counting chamber (Superior Marienfell – Germany) and the concentration of inoculum was adjusted to 10^6 conidia/ml. Inoculation of plants were done 4 weeks after sowing by spraying leaves till run-off followed by covering inoculated plants with transparent plastic bags for 18 hours (Mchau et al., 1998). In the control treatment, sterile water was sprayed on the plants in place of the conidium suspension. There were four plants per treatment replicated three times and the buckets were arranged in a Completely Randomized Design (CRD).

Assessment of disease was done at 7 and 14 days after inoculation. A plant was considered positive for infection if a clearly distinguishable scab lesion developed, negative where no infections were developed while infections which were spotting but with no clearly identifiable scab lesions were designated as plus or minus (±) (Hyun et al., 2009). Disease severity was measured using a scale of 0-3 where 0 = no visible symptoms; 1 = very small pinprick type; 2 = small dark brown lesions with no chlorosis; and 3 = pale brown lesions surrounded by a chlorotic halo and with some distortion of the lamina (Mchau et al., 1998). Where there were any doubts about the identity of the symptoms, re-isolation was made from symptomatic plants inoculated with isolates.

Table 2. List of 20 selected genotypes used for pathogenicity test and their reported resistance ratings

Genotype	Resistance rating[a]	Days to 50% flowering[b] (days)	Yield potential[c] (t/ha)
Accession 12 * Secow 2W	MR	48	1.36
Accession 23	S	47	0.93
Accession 23 * Accession 12	R	50	1.32
Alegi	MR	49	1.42
NE 15	R	49	1.63
NE 23	R	46	1.31
NE 31	R	48	1.46
NE 48	MR	48	1.43
NE 50	MR	49	1.45
NE 70	R	49	1.25
Secow 1T	MR	50	1.59
Secow 5T	MR	49	2.05
Sun shine	S	49	0.72
WC 10	MR	46	1.61
WC 17	R	48	0.87
WC 29	S	51	0.89
WC 35B	R	50	1.40
WC 36	S	49	1.21
WC 66	MR	51	1.43
WC 67	MR	49	1.42

Note. [a] resistance rating by Afutu et al. (2016b): R = Resistant, MR = Moderately Resistant, S = Susceptible; [b] average data from two locations in 2014 season A (April-July); [c] average yield obtained from two locations (Afutu et al., 2016b).

Percentage pathogenic reactions of genotypes to isolates were calculated by expressing the number of isolates that caused clear scab disease symptoms (with the + symbol) over the five isolates (excluding the control treatment) and multiplied by 100 while percentage pathogenicity of isolates were estimated by expressing the number of genotypes with clear scab disease symptoms (with the + symbol) over the 20 genotypes and multiplied by 100. To estimate pathogenicity values for each isolate, pathogenicity codes (0-2) were assigned to the three symbols used in designating the presence or absence of scab disease symptoms (Hyun et al., 2009), where, 0 = "-" (no infections developed), 1 = "±" (for infections which were spotting but no clearly identifiable

scab lesions), and 2 = "+" (clearly distinguishable scab lesions). Following inoculation with the isolates, scab disease incidence was expressed as the percentage of infected plants and the incidence data was transformed using arcsine transformation of arcsine percentage (K. A. Gomez & A. A. Gomez, 1984) after a Kurtosis-Skewness test showed a significant deviation from the normal. Mean severity scores were estimated using Microsoft Excel and the means obtained were used to calculate area under the disease progress curve (AUDPC) at 7 and 14 days after inoculation for each cowpea genotype in Microsoft Excel using the formula of Campbell and Maddden (1990):

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2}\right) (t_{i+1} - t_i) \quad (1)$$

Where "t" is the time of each reading, "y" is the percent of affected foliage at each reading (severity score) and "n" is the number of readings. The variable "t" represents days after inoculation. The transformed incidence data, mean severity and AUDPC calculated were analysed for variation (ANOVA) using Genstat edition 14 (Payne et al., 2011) and the disease incidence, severity and AUDPC means were separated using Fisher's protected Least Significant Difference (LSD) test at $P < 0.05$.

2.3 Growth Rate

The growth rate of the fungus was measured by culturing the isolates on commercial preparations of Potato Dextrose Agar (PDA) (Difco, Detroit) amended with Streptomycin Sulphate (1.5 g/L) and rose bengal (0.0025 g/L of agar). Radial growth was measured for a total of 42 isolates selected across the 14 districts and from different morphological groups. Three isolates were selected from each district (one from each Sub-County) based on the farm with the highest incidence and severity within a Sub-County (Afutu et al., 2016a). Mycelial disks (5 mm) from 7-day-old fungal cultures were transferred to the centre of 90 mm diameter Petri plates and sealed with Parafilm (Miyashira et al., 2010; Turkkan & Erper, 2014). Two separate petri dishes were prepared for each isolate representing two replicates and the dishes were incubated under 12 hours of alternating light and darkness at 25 ^{o}C (±1 ^{o}C) for a maximum of 14 days.

Two straight lines were drawn perpendicular to each other on the bottom of the petri dishes ensuring that the crossing point coincided with the centre of the 5 mm fungi disc plugs (Figure 1) and radial growth measurements were recorded following the procedure of Miyashira et al. (2010) with slight modifications by measuring growth rates on daily basis instead of weekly. Radial growth measurements were taken on daily basis until the fungus mycelia reached the walls of a dish or for a maximum of 14 days and the daily recordings of growth were expressed as mm/day.

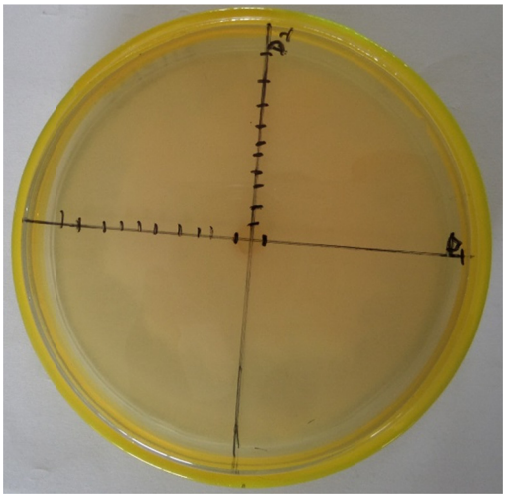

Figure 1. Radial growth rate measurement for scab fungus isolates on PDA

Analysis of variance of fungus growth rate for different time intervals (i.e. 7, 10 and 14 days) after inoculation of media and days to full coverage (DTFC) of petri dish was performed in Genstat edition 14 (Payne et al., 2011). For more accuracy, the maximum likelihood method was used (Payne et al., 2003) in Genstat analysis for a Chi-square test for independence or association of growth rate data with the Districts and Agro-ecological zones from which isolates were obtained.

3. Results and Discussion

3.1 Morphological Characterization of Pathogen

There was a wide variation in the isolates. Morphological characterization of the 495 isolates based on the amount, nature, colour, depth and rate of mycelia growth, nature or shape of conidia, number of septations and the colour of the base of petri dishes when inverted yielded six (6) morphological groupings (A-F) (Table 3). Each of these groups had morphological structures similar to those described by Barnett and Hunter (1987) and (Ayodele & Kumar, 2014) but with slight variations in the parameters, hence, the different morphological groupings.

Table 3. Description and morphological characterization of 495 *Sphaceloma* sp. isolates from Uganda

Group	Isolates[a]	Source of isolates		Total isolates %	Characteristics of group
		Leaf	Pod		
A	70 Isolates: TR175P; NB425P*; AR439L; AR440L; KU88L; LR369L; LR372L; PA132P; PD217L; PD218L; PD219L; PD220L; PD225L; PD226L; PD227L; PD228L; PD233L*; PD234L; YU471L; YU472L; YU481L*; YU482L; YU483L; YU484; YU485L; YU486L; YU487L; YU488L; YU489L; YU490L; YU493L; YU494L; YU497L; YU498L; KT253L; KT254L; KT255L; KT256L; KT257L; KT258L; KT259L*; KT260L; KT261L; KT262L; KT263L; KT264L; KT265L; KT266L; KT267L; KT268L; KT269L; KT270L; KT271L; KT272L; KT273L*; KT274L; KT275L; KT276L; KT277L*; KT278L; KT279L; KT280L; KT281L; KT282L; KT283L; KT284L; KT285L; KT286L; KT287L; KT288L.	67	3	14.1	➢ Very scanty mycelia ➢ Entirely grey coloured and submerged in the medium ➢ Conidia hyaline with 0-2 septation ➢ Underneath cultures greyish-brown ➢ Slow growing isolates
B	35 Isolates: ST13P*; LR395L; LR396L; AR445L; AR446L*; AR447L; AR448L; AR449L; AR450L; AR451L; AR452L; AR453L; AR454L; AR455L; AR456L; YU469L*; YU470L; YU473L; YU474L; YU475L; YU476L; YU477L; YU478L; YU479L; YU480L; YU491L; YU492L; YU495L; YU496L; YU499L; YU500L; YU501L*; YU502L; YU503L; YU504L.	34	1	7.1	➢ Scanty pale coloured mycelia ➢ Brownish through pink to black patches of mycelia at the centre ➢ Mycelia submerged in the medium ➢ Hyaline conidia with 1-4 saptation ➢ Underneath cultures peach-orange to pink ➢ Slow growing isolates
C	72 Isolates: ST5L; ST6P; ST9L; ST10L; ST11L; ST12L; ST14L; ST15L; ST16L; ST19L; ST21L; ST22L; ST23L; ST25L*; ST26L; ST27L; ST28L; ST29L; ST30L; ST31L; ST32L; ST33L; ST34L; ST35L; ST36L; PA121L; PA124L; KU76L; KU77L; KU78L; KU79L; KU81P; KU82P;	62	10	14.5	➢ Hyaline fresh mycelia growth ➢ Old mycelia growths redwood to red-brown

Table 3. Continued

Group	Isolates[a]	Source of isolates		Total isolates %	Characteristics of group
		Leaf	Pod		
C	KU84P; KU86P; KU93P; KU94L; KU103L; SE41L; SE42L; SE43L; SE49L; SE50L; AR433L*; AR441L; AR442L; AR443L*; AR444L; LR361L; LR362L; LR363L; LR364L; LR365L*; LR366L; LR367L; LR368; LR371L; LR373L; LR374L; LR375L; LR376L; LR379L; LR380L; LR381L; LR382L; LR383L*; LR384L; LR388L; TR190P; TR173P; TR177P; TR178P.				with black patches ➢ Submerged scanty mycelia ➢ Conidia hyaline with 1-2 septation ➢ Pink to red-brown underneath cultures ➢ Slow growing Isolates
D	80 Isolates: SE48L; LR393L; LR394L; AR465L*; AR466L; AR467L; AR468L; TR147L; TR158P; TR166L; PA123L; PD229L; PD230L; PD231L; PD232L; PD235L; PD236L; PD239L; PD240L; KU85P; KU101L; KU104L; KU105L*; AM181L; AM182L; AM183L*; AM184L; AM185L; AM186L; AM187L; AM188L; AM189L; AM190L; AM191L; AM192L; AM193P; AM194P; AM195P; AM196P; AM197L; AM198L; AM199L; AM200L; AM201L*; AM202L; AM203L; AM204L; AP289L; AP290L; AP291L; AP292L*; AP294P; AP297L; AP298L; AP299L; AP300L; AP301L; AP302L; AP303L; AP304L; AP305P; AP306P; AP307P; AP308P; AP309L*; AP310L; AP311L; AP312L; AP313L; AP314L; AP315L; AP316L; AP317L*; AP318L; AP319L; AP320L; AP321L; AP322L; AP323L; AP324L.	65	15	16.2	➢ Grey fresh mycelia growths ➢ Old growths brown coloured ➢ Submerged scanty mycelia ➢ Conidia hyaline with 1-2 septation ➢ Greyish-brown to red-brown underneath cultures Fast growing isolates
E	36 Isolates: PD241L, PD242L; PD243L; PD244L; PD245L; PD246L; PD247L*; PD248L; PD249L; PD250L; PD251L; PD252L; KU87L; KU95P; KU80L; ST24L; TR148P; LR385L; LR386L; LR387L; LR389L*; LR390L; LR391L; LR392L; AM205P*; AM206P; AM207P; AM208P; AM209P; AM210P; AM211P; AM212P; AM213P; AM214P; AM215P; AM216P.	22	14	7.3	➢ Abundant hyaline mycelia ➢ Centre Peach coloured ➢ Fresh hyaline mycelia growths ➢ Mycelia submerged in medium ➢ Conidia hyaline with 1-4 septation ➢ Grey to peach underneath cultures ➢ Fast growing isolates

Table 3. Continued

Group	Isolates[a]	Source of isolates		Total isolates %	Characteristics of group
		Leaf	Pod		
F	202 Isolates: ST1L; ST2L*; ST3P; ST4L; ST7P; ST8P; KU73L; KU74L*; KU83P; KU89L; KU90L; KU91L*; KU92L; KU95P; KU96L; KU97L; KU98P; KU99P; KU100L; KU102L; KU104P; KU106P; KU107P; KU108L; SE37L; SE38L*; SE39L; SE40L; SE44L; SE45L; SE46L; SE47L; SE51L; SE52L; SE53L; SE54L; SE55L; SE56L; SE57L*; SE58L; SE59L; SE60L; SE61L; SE62L; SE63L; SE64L; SE65L; SE66L*; SE67L; SE68L; SE69L; SE70L; SE71L; SE72L; AR434L; AR435L; AR436L; AR437L; AR438L; AR457L; AR458L; AR459L; AR460L; AR461L; AR462L; AR463L; AR464L; TR145P*; TR146P; TR149L; TR150P; TR151P; TR152P; TR153P; TR154P; TR155L; TR156P; TR157P; TR159P; TR161L; TR162P*; TR163P; TR164L; TR165P; TR167P; TR168P; TR169L; TR170L; TR171L*; TR172L; TR174P; TR179P; TR180L; NB397L; NB398L; NB399L*; NB400L; NB405L; NB406L; NB407L; NB408L; NB413L; NB414L*; NB415L; NB416L; NB417L; NB418L; NB419L; NB420L; NB421L; NB422L; NB423L; NB424L; NB426L; NB427L; NB428L; NB429L; NB430L; NB431L; NB432L; PD221L; PD222L; PD223L; PD224L*; PD237L; PD238L; PA109L; PA110L*; PA111L; PA112L; PA113L; PA114L; PA115L; PA116L; PA117L; PA118L; PA119L; PA120L; PA122L; PA125L; PA126L*; PA127L; PA128L; PA129L; PA130L; PA131L; PA133L; PA134L; PA135L; PA136L; PA137L*; PA138L; PA139L; PA140L; PA141L; PA142L; PA143L; PA144L; DK325L; DK326L; DK327L; DK328L; DK329L; DK330L; DK331L; DK332L; DK333L; DK334L*; DK335L; DK336L; DK337L; DK338L; DK339L; DK340L; DK241L; DK342L*; DK343L; DK344L; DK345L; DK346L; DK347L; DK348L; DK349L; DK350L; DK351L*; DK352L; DK353L; DK354L; DK355L; DK356L; DK357P; DK358P; DK359L; DK360L; NB409L; NB410L; NB411L; NB412L; NB401L; NB402L; NB403L; NB404L.	169	33	40.8	➢ Abundant and hyaline mycelia ➢ Both old and fresh growths entirely white ➢ Mycelia submerged in medium ➢ Conidia hyaline with 1-4 septation ➢ White underneath cultures ➢ Fast growing isolates
Total	495 Isolates	419	76	100	-

[a] Isolates with the asterisks sign attached (*) constitute isolates selected from the different groups for radial growth rate measurements.

The amount of mycelia produced by the isolates ranged from very scanty (Group A = 14.1%) through scanty (Groups B, C and D = 37.8%) to abundant (Groups E and F = 48.1%) while the colour of colonies produced varied widely from white (Figure 2F), pale (Figure 2A), white and peach (Figure 2E), through to pink with red-brown to brownish-black pigmentations (Figures 2B, 2C and 2D) confirming earlier reports that colony colour on PDA was found to be extremely variable even within the same isolates being differently pigmented under the same growth conditions (Zeigler & Lozano, 1983). The variable colour of the colonies observed in this study, including the dark to black pigmentations and the consistently high pigmentation has also been reported in some of the closely related species such as *Elsinoe fawcettii*, *E. australis* and *Sphaceloma fawcettii*, the causal organisms of scab diseases of citrus (Timmer et al., 1996).

According to Barnett and Hunter (1987), fungal hyphal cells vary in their size, colour and in their extracellular matrix when present, however, since hyphae among different kinds of fungi are more alike than different, they usually cannot be used as a differentiating character. Zeigler and Lozano (1983) also reported that different *Sphaceloma* sp. proved impossible to distinguish using colony morphology and colour alone. In this study, delineation of isolates into the different morphological groups were further based on the features of conidia belonging to the genus *Sphaceloma* as described by Alvarez et al. (2003). Therefore, based on other features apart from those of the fungal mycelia, 70 (14.1% Group A), 152 (30.7% Groups C and D) and 273 (55.2% Groups B, E and F) of the isolates had 0-2, 1-2, and 1-4 septa respectively.

The number of septa of species belonging to the genus *Spahceloma* was reported to be 0-2 (Zeigler & Lozano, 1983) and 0-3 (Alvarez et al., 2003; Ayodele & Kumar, 2014), however, a greater number of the Ugandan isolates (273 representing 55.2% belonging to Groups B, E and F) had 1-4 septa, thus suggesting that these isolates may belong to different species within the genus *Sphaceloma*. While some of the isolates (about 37.6%) had scanty and submerged mycelia as described by Ayodele and Kumar (2014), most of the isolates (Groups E and F = 48.1%) had abundant mycelia but were also submerged suggesting a variation within the genus. To ensure more robust grouping of isolates, the colour of the under-side (reverse side) of petri dishes of cultures when inverted as applied in morphological characterization of *Colletotrichum acutatum* isolates causing anthracnose of lupins (Talhinhas et al., 2002), was used as an additional parameter to delineate the isolates into groups. The colour of the base of petri dishes when inverted varied from white through grey to peach, and greyish-brown to red-brown (Table 3).

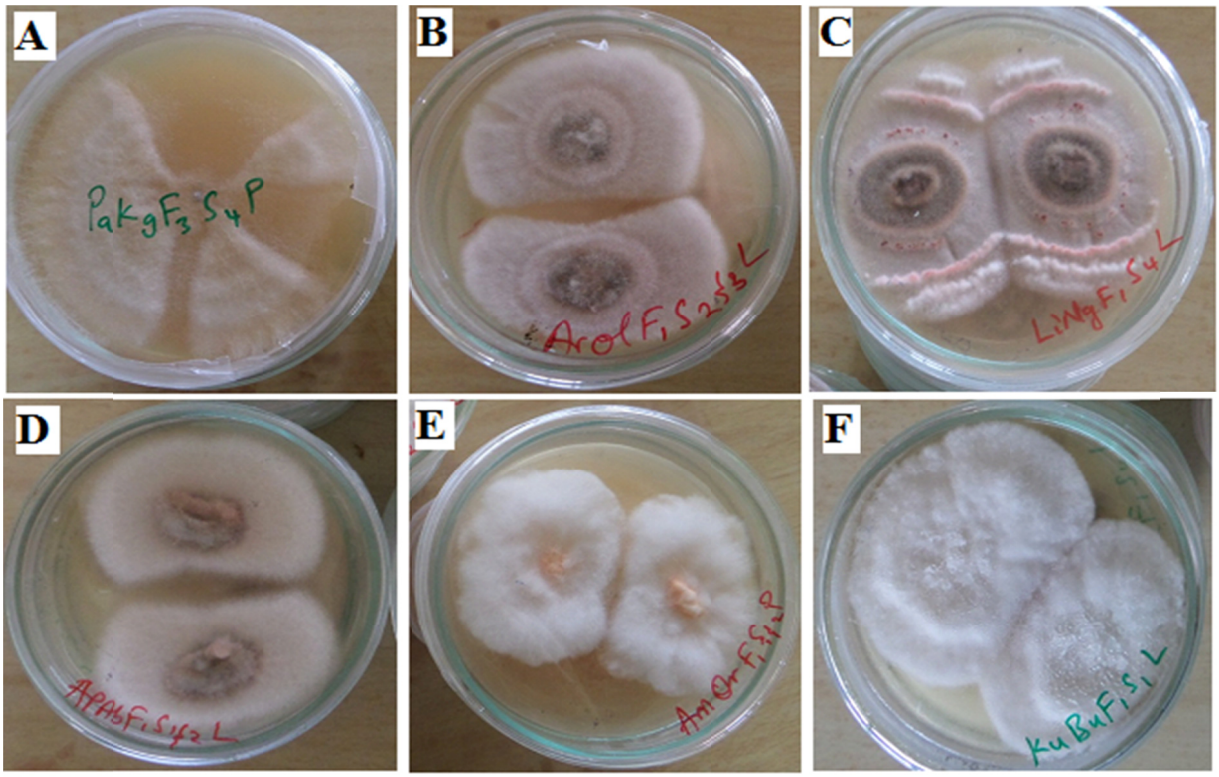

Figure 2. Colony characters of the different *Sphaceloma* sp. groups on PDA

Table 4. Pathogenicity of 5 *Sphaceloma* sp. isolates on 20 selected cowpea genotypes

Genotype	RL[a]	Pathogenicity of isolate						Pathothogenic reaction to isolate (%)[b]	Mean		
		Control	KU78L	TR171L	KT259L	PD232L	AR446L		Incidence	Severity	AUDPC
Accession 12 × Secow 2W	MR	-	+	+	±	+	±	3 (60.00)	52.8	2.3	13.42
Accession 23	S	-	-	+	+	±	+	3 (60.00)	44.4	1.8	11.86
Accession 23 × Accession 12	R	-	+	±	-	-	-	1 (20.00)	23.6	1.5	9.92
Alegi	MR	-	±	-	+	±	-	1 (20.00)	31.9	1.7	10.69
NE 15	R	-	±	+	±	-	-	1 (20.00)	20.8	1.5	8.75
NE 23	R	-	-	+	±	-	±	1 (20.00)	22.2	1.7	10.69
NE 31	R	-	±	-	-	-	-	0 (0.00)	11.1	1.2	7.97
NE 48	MR	-	-	+	+	-	+	3 (60.00)	41.7	1.8	11.47
NE 50	MR	-	±	+	+	+	±	3 (60.00)	55.6	2.2	14.19
NE 70	R	-	±	-	-	-	-	0 (0.00)	8.3	0.9	7.39
Secow 1T	MR	-	+	+	±	-	+	3 (60.00)	36.1	1.9	11.86
Secow 5T	MR	-	-	±	+	-	-	1 (20.00)	18.1	1.4	9.53
Sun shine	S	-	+	+	±	+	+	4 (80.00)	58.3	3.4	17.69
WC 10	MR	-	-	+	-	+	+	3 (60.00)	33.3	1.8	10.69
WC 17	R	-	±	+	+	-	±	2 (40.00)	37.5	1.8	11.08
WC 29	S	-	+	+	±	+	+	4 (80.00)	52.8	2.3	14.00
WC 35B	R	-	-	±	+	-	-	1 (20.00)	22.2	1.4	9.33
WC 36	S	-	±	+	+	+	±	3 (60.00)	51.4	2.2	13.03
WC 66	MR	-	+	-	+	-	-	2 (40.00)	23.6	1.5	9.53
WC 67	MR	-	±	+	-	+	+	3 (60.00)	36.1	1.8	11.86
Percentage Pathogenicity[c]		-	30	65	45	35	35				
Pathogenicity value[d]		0	20	29	24	16	19				
Pathogenicity group[e]		-	1	3	2	1	1				
$LSD_{(0.05)}$									7.6	0.7	2.7
S.E.									11.6	1.1	1.0
CV (%)									34.0	63.1	36.4

Note. [a] Resistance rating based on Afutu et al. (2016b); RL = Resistance level; R = resistant; MR = moderately resistant; S = susceptible;

[b] Pathogenic reaction of genotypes = number of isolates that caused clearly distinguishable scab lesions on genotypes out of the five isolates × 100;

[c] Percent Pathogenicity of isolates = number of genotypes with clearly distinguishable scab lesions caused by each isolate out of the 20 genotypes × 100;

[d] Pathogenicity value = summation of pathogenicity codes 0-2 based on the symptomatic effects of each isolate;

[e] Pathogenicity groups: Isolates were separated into three groups based on the mean disease incidence, severity, AUDPC and the pathogenicity of isolates on the 20 inoculated cowpea genotypes, using Ward's cluster analysis (Alvarez et al., 2003) with 94% level of confidence.

3.2 Pathogenicity of Selected Isolates

The results of pathogenicity test of five selected *Sphaceloma* sp. isolates conducted on the 20 cowpea genotypes are presented in Table 4. The inoculation procedure proved satisfactory because susceptible genotypes were found to be consistently infected by the isolates though to varying degrees. Characteristic scab disease symptoms were observed on leaves of most infected genotypes by the time of the first observation (7 days after inoculation). No scab disease symptoms were observed on stems even 14 days after inoculation of genotypes. Symptoms

produced on leaves included necrotic spots which were only visible on leaf surfaces by the time of first observation but became visible on both leaf surfaces by the second observation. Neither perforations nor leaf distortions were observed as would normally be seen on scab infected plants because the period of 14 days was too short a time for such advanced symptoms to be observed.

Ward's cluster analysis of the 20 genotypes based on the pathogenicity of the isolates, mean incidence, severity and AUDPC, grouped the genotypes into 4 significant clusters (K = 4) (Figure 3). Cluster 1 consisted of 5 genotypes (Accession 12 × Secow 2W, NE 50, WC 29, WC 36 and Sun shine) with mean incidence, severity and AUDPC ranging from 51.4-58.3%, 2.2-3.4, and 13.0-17.7 (Table 4) respectively while cluster 2 consisted 7 genotypes (Accession 23, NE 48, Secow 1T, WC 10, WC 17, WC 66 and WC 67) with means of 23.6-44.4%, 1.5-1.9 and 9.5-11.9 for incidence, severity and AUDPC respectively (Table 4). Cluster 3 comprised 6 genotypes (Accession 23 × Accession 12, Alegi, NE 15, NE 23, Secow 5T and WC 35B) with mean incidence, severity and AUDPC ranges of 18.1-31.9%, 1.4-1.7 and 8.8-10.7 respectively while cluster 4 comprised 2 genotypes (NE 31 and NE 70) with means of 8.3-11.1%, 0.9-1.2 and 7.4-8.0 for the three disease indexes respectively (Table 4). Apart from cluster 4 which consisted of only genotypes rated resistant to the pathogen, the remaining three clusters consisted genotypes with different resistance levels (Figure 3) which implies that these genotypes responded or reacted similarly to infection by the isolates. NE 31 and NE 70 showed a broader spectrum of resistance to infection by all the isolates, thus, confirming their resistance rating reported by Afutu et al., (2016b), hence making them good parents for the breeding program to develop cultivars that would have a wide horizontal resistance to the scab disease. According to Acquaah (2007), evolution of new races due to selection pressure against any specific race is absent under horizontal resistance, and provides protection against a wide range of races of a pathogen, hence, is less susceptible to being overcome by a new race making this type of resistance more stable.

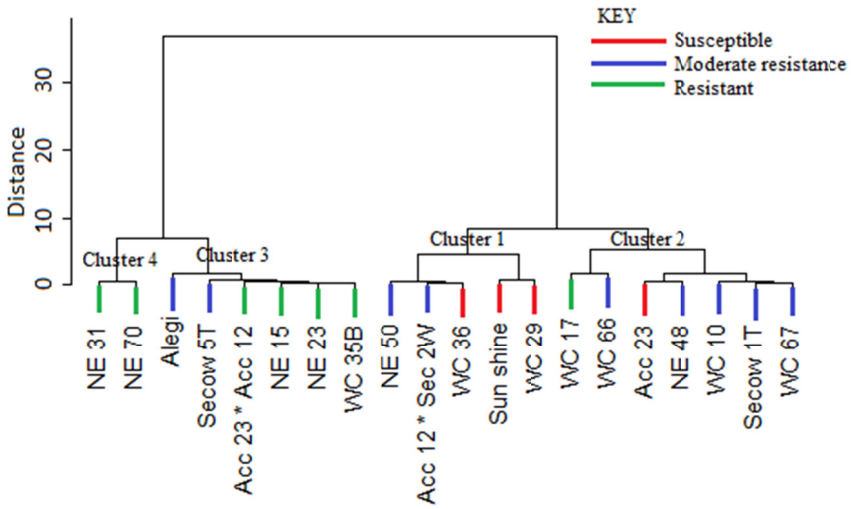

Figure 3. Ward's cluster dendogram of the 20 cowpea genotypes based on disease incidence, severity, AUDPC and pathogenicity of isolates

There were variable levels of pathogenicity among the isolates (percentage pathogenicity) and different responses of genotypes to isolates (pathogenic reaction to isolates) (Table 4). The variable pathogenicity levels as shown in Table 4 proved to be significantly different when the data on pathogenicity were subjected to the analysis of variance procedure (Table 5). There were highly significant differences ($P < 0.001$) in virulence of isolates for disease incidence, severity and AUDPC (Table 5). The results of the pathogenicity tests carried out in the screen house corroborated the findings from disease evaluations conducted under field pressure conditions at different locations (Afutu et al., 2016b) as cowpea genotypes rated resistant to the disease were found to show very low percentage pathogenic response (0-20%) to the isolates. Genotype by isolate interaction was also found to be significant ($P < 0.001$) meaning that the resistance levels of the genotypes to the pathogen had effects on the level of virulence or pathogenicity of the isolates. This was expected because the genotypes selected for the

test have been reported to have different levels of resistance when evaluated for resistance to the pathogen under natural conditions in the field and under different environments (Afutu et al., 2016b).

Table 5. Combined Analysis of variance for scab disease incidence, severity and AUDPC caused by isolates 5 isolates and a control treatment on 20 selected cowpea genotypes.

Source of Variation	df	Mean square			Pr > F
		Incidence	Severity	AUDPC	
Genotypes	19	4131.9***	4.7***	105.2***	0.001
Isolate	5	22580.9***	10.4***	294.7***	0.001
Genotype × Isolate	95	2594.8***	2.4***	51.9***	0.001
Error	238	134.1	1.3	16.8	

Note. *** = significant at P < 0.001.

Using Ward's cluster analysis (94% confidence level), the isolates were separated into three clusters (Figure 4) based on the mean incidence, severity, AUDPC and pathogenicity on the 20 genotypes. Cluster 1 comprised three isolates (KU78L, PD232L and AR446L), all of which were obtained from different agro-ecological zones with mean incidence, severity and AUDPC ranging from 30.0-40.4%, 1.8-2.0, and 11.1-12.0 (Table 6) respectively while clusters 2 and 3 were formed by isolates KT259L and TR171L respectively. The mean incidence, severity and AUDPC for cluster 2 were 45.4%, 2.0 and 12.1 respectively while cluster 3 had means of 57.1%, 2.2 and 13.5 for the three traits (Table 6).

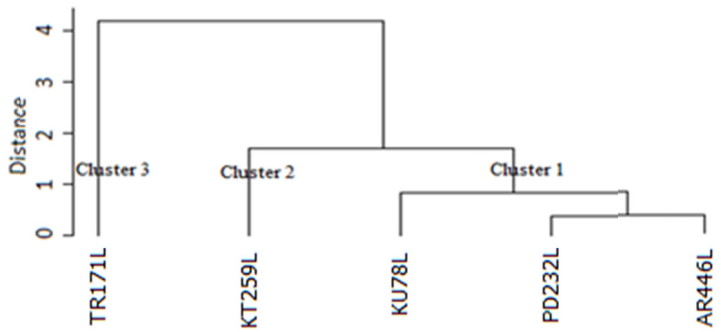

Figure 4. Ward's cluster dendogram of five isolates based on disease incidence, severity, AUDPC and pathogenicity of isolates

3.3 Growth Rate

The results of radial growth rate (mm/day) for 42 selected isolates are presented in Table 7. There were significant variations (P < 0.001) among the isolates, Districts, morphological groups and agro-ecological zones for radial growth rates at 7, 10, and 14 days after inoculation of media. The number of days to full coverage (DTFC) was significantly variable among isolates and Districts (P < 0.001) and also significantly different (P < 0.01) among the different morphological groupings and agro-ecological zones from which the isolates were obtained. The variability in radial growth rate of the isolates were not surprising as this could be accounted for by the fact that the isolates were selected from different morphological groups (indicated by asterisks '*' in Table 3). Among the traits used for delineating isolates into the different morphological groups was the amount of mycelia produced by the isolates in culture, ranging from scanty to abundant.

Table 6. Mean scab disease incidence, severity and AUDPC caused by 5 isolates and a control treatment on 20 cowpea genotypes

Isolate	Mean		
	Incidence	Severity	AUDPC
Control	0.0	1.0	7
KU78L	40.4	1.9	11.8
TR171L	57.1	2.2	13.5
KT259L	45.4	2.0	12.1
PD232L	31.7	1.8	11.1
AR446L	30.0	2.0	12.0
LSD (0.05)	4.2	0.4	1.5
S.E.	11.7	1.1	0.6
CV (%)	34.3	63.2	5.2

The use of radial growth rate has been shown to be a good measurement approach to differentiate isolates of the same species tested on similar types of media and the approach makes it easier to compare different data obtained within the same experiment or with other experiments to differentiate one isolate from the other or determine which media is best for growth of isolates (Miyashira et al., 2010).

Table 7. Combined Analysis of variance for radial growth rate of 42 *Sphaceloma* sp. isolates for different time intervals

Source of variation	df	Mean square				Pr > F
		Day 7[a]	Day 10[b]	Day 14[c]	DTFC[d]	
Isolate	41 (41)	331.8 (92.8)***	405.8 (139.7)***	308.0 (81.5)***	8.8 (2.3)***	0.001
District	13 (69)	661.0 (127.8)***	795.6 (174.2)***	581.5 (121.8)***	18.4 (3.2)***	0.001
MG	5 (77)	900.8 (167.6)***	1066.7 (221.2)***	528.1 (173.1)**	18.4 (4.7)**	0.001
AEZ	2 (80)	1875.0 (170.7)***	2450.4 (218.3)***	1586.0 (159.9)***	33.5 (4.9)**	0.001

Note. Figures in parenthesis are error values; MG = Morphological group; AEZ = Agro-ecological Zone; *** = significant at $P < 0.001$.

[a, b] and [c] represents radial growth rates measured at 7, 10 and 14 days respectively after inoculation of media (PDA) with 5 mm diameter disc plugs of isolates.

[d] = days to full coverage of entire surface of the 90 mm petri dishes.

There was an association between radial growth rate (mm/day) of *Sphaceloma* sp. isolates and the District and agro-ecological zones from which isolates were obtained. The results of chi-square test of independence to test the hypotheses that radial growth rate of *Sphaceloma* sp. isolates were independent of Districts of origin and agro-ecological zones yielded the following test statistic values ($\chi^2 = 67.94$, df = 26, and $P < 0.001$) and ($\chi^2 = 21.08$, df = 4, and $P < 0.001$) for Districts and agro-ecological zones respectively (Table 8) hence the rejection of the two null hypotheses that growth rate of *Sphaceloma* sp. isolates was independent of districts and agro-ecological zones of origin. Out of the chi-square statistic of 67.94 for districts, isolates from Apac district contributed a margin of 14.869, out of which most (8.680) was due to fast growth rate which means that most of the isolates from the Apac district were fast growing, and covered the entire petri dish by the 8th day. Isolates from two districts, *viz*., Kitgum and Yumbe showed similar growth rates, albeit from different ecological zones, as indicated by their contributions (9.839) each to the chi-square statistic. Also, out of the chi-statistic value of 67.94, slow growth rate contributed most (28.419) indicating that most of the *Sphaceloma* sp. isolates were slow growing (took > 14 days to cover the entire petri dish).

Table 8. Combined chi-square test of independence for growth rate of *Sphaceloma* sp. isolates with Districts and Agro-ecological zones from which isolates were obtained

District growth rate[a]					Regional growth rate[a]				
District	Slow	Moderate	Fast	Margin	AEZ[b]	Slow	Moderate	Fast	Margin
Amuria	1.016	1.342	0.571	2.929	EAEZ	4.282	4.767	3.429	12.478
Apac	6.143	0.046	8.680	14.869	NESG	3.919	2.697	0.433	7.049
Arua	0.427	0.046	1.077	1.550	NWSG	0.427	0.046	1.077	1.550
Dokolo	1.899	1.661	0.571	4.131					
Kitgum	6.143	3.125	0.571	9.839					
Kumi	0.002	0.046	0.571	0.619					
Lira	0.002	0.046	0.571	0.619					
Nebbi	2.178	5.286	0.571	8.035					
Pader	0.002	0.046	0.571	0.619					
Palisa	1.016	1.342	0.571	2.929					
Serere	2.178	5.286	0.571	8.035					
Soroti	1.016	1.342	0.571	2.929					
Tororo	0.256	0.171	0.571	0.998					
Yumbe	6.143	3.125	0.571	9.839					
Margin	28.419	22.910	16.614	67.943		8.628	7.511	4.938	21.077
χ^2	67.94				21.08				
df	26				4				
P	< 0.001				< 0.001				

Note. [a] Slow, moderate and fast growth rates implies isolates took 1-8, 9-14 and > 14 days respectively to grow to cover entire surface of petri dish (90 mm diameter); b AEZ = Agro-ecological zone, EAEZ = Eastern Agro-ecological zone, NESG = North Eastern Savannah Grassland, NWSG = North Western Savannah Grassland.

On the other hand, out of the test statistic value of 21.08 for the agro-ecological zones, isolates from the Eastern Agro-ecological Zone (EAEZ) contributed the most margin (12.478) out of which a greater part of it was due to moderate growth rate implying that most of the isolates from the EAEZ had moderate growth rate, thus, took between 9-14 days to cover the entire (90 mm) petri dish. On the whole, the observation that most of the *Sphaceloma* sp. isolates were slow (> 14 days) in growth rates (28.419 margin out of $\chi^2 = 67.943$) as shown in the chi-square test between growth rate and districts of origin, was confirmed by the chi-square test of growth rate being independent of the agro-ecological zones of origin.

Thus, most of the margin (8.628 out of $\chi^2 = 21.08$) was contributed by slow growth rate. This finding that the isolates were mostly slow growing is entirely consistent with earlier reports of studies on the genus *Sphaceloma* and its related genus *Elsinoe* (Zeigler & Lozano, 1983; Timmer et al., 1996).

4. Conclusion

The study revealed a wide morphological variation in the scab fungus (*Sphaceloma* sp.) occurring in Uganda with the isolates being grouped into six morphological and three pathogenicity groups. Growth rate of the fungus was found to be dependent on the District and Agro-ecological zones of origin and mostly slow growing. NE 31 and NE 70 cowpea genotypes showed broader spectrum of resistance to the isolates and could therefore be used to introgress resistance in a breeding programme to develop cultivars with wide horizontal resistance to the scab disease.

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