PATHOGENIC VARIABILITY IN *PHAEOISARIOPSIS GRISEOLA* (SACC.) FERR. AND RESISTANCE OF *PHASEOLUS VULGARIS* L. TO ANGULAR LEAF SPOT.

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted with our approval as University supervisors.

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DEDICATION

To my parents

Mr Obadiah Wagara

and

Mrs Joyce Nyambura

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LIST OF ABBREVIATIONS

ALS - Angular leafspot

cv - Cultivar

GLP - Grain legume project

BLDA - Bean leaf dextrose agar

R - Resistant

S - Susceptible

Abstract

Pathogenic variability in *Phaeoisariopsis griseola* Sacc. was studied using eighteen isolates obtained from naturally infected bean (*Phaseolus vulgaris* L.) plants collected from fields with varying ecological conditions in Kenya. Each isolate was used to inoculate thirty bean differential cultivars/lines. The 18 isolates were grouped into 15 different races based on their differential reactions on the 30 bean differential cultivars/ lines. The isolates interacted differentially with the host bean cultivars and some of the bean cultivars earlier reported to be resistant to angular leaf spot disease were susceptible to at least one of the Kenyan isolates. Some isolates were more aggressive than others and induced symptoms as early as 6 days after inoculation while others took up to 19 days to induce the first symptoms on the susceptible cultivars.

The range of angular leafspot symptoms induced by *P. griseola* isolates on bean cultivars was wide and ranged from small angular lesions clearly delimited by veins and veinlets to coalesced aggregate lesions of greater dimensions. This variation could not be attributed to either the isolate or cultivar used but it further supported the fact that pathogenic variability does exist in *P. griseola*.

Growth of *P. griseola* isolates on BLDA and the variation in conidial sizes were studied to determine whether any differences observed were race specific. The variations noted were not race-specific and these parameters were found to be of no significance in race typing the pathogen.

Fungal development and the associated cellular reactions in three bean cultivars; a highly susceptible cultivar (Rosecoco-GLP-2), an intermediate resistant line (M29) and a resistant line (M26) inoculated with *P. griseola* isolate PG18 were studied. Conidia of *P. griseola* germinated 4 hours after inoculation in all the three cultivars/lines but germination in line M26 was slightly inhibited. Penetration of the host tissues was either direct or through the stomata. A prolonged biotrophic phase was noted in cv Rosecoco-GLP-2 and line M29 where lesions appeared on the 6^{th} and 10^{th} day respectively whereas tissue browning was noted in line M26 four days after inoculation. Cell disintegration resulting into lesion development occurred 6 days after inoculation in cv Rosecoco-GLP-2 and line M29 but the cells in line M26 were still intact.

Colonization of host tissues by *P. griseola* was extensive in cv Rosecoco-GLP-2 and line M29 but was restricted in line M26. The fungus only colonized the lower epidermal and spongy mesophyll cells. Sporulation in cv Rosecoco-GLP-2 was heavy and occurred 10 days after inoculation whereas in line M29, it was limited and occurred 5 days later but was totally inhibited in line M26. The number of synnemata per lesion and number of conidiophores per synnema were significantly ($P=0.01$) more in cv Rosecoco-GLP-2 than in line M29. Thus probably resistance in bean line M26 to *P. griseola* is due to inhibited spore germination, tissue colonization and sporulation whereas the intermediate response in line M29 is due to delayed and limited sporulation of the fungus.

CHAPTER ONE

1.0 INTRODUCTION

1.1. Bean production in Kenya

Bean (*Phaseolus vulgaris*) is the most important legume in Kenya. Approximately 630,000 hectares of land is under beans with yields of about 410,000 metric tonnes (Anon, 1994). Beans are grown from between 900-2700 metres above sea level but do best at medium altitude areas of 900-2100M (Acland, 1971). They grow best in well drained soils with high nutrient content and a pH of 5.2. Beans do not grow well below 600M above sea level because of the high temperatures which affect pod filling. The major bean growing areas are Western, Eastern and Rift Valley provinces but beans are also widely grown in Nyanza and Central provinces (Anon, 1994). At the Coast province, beans are grown at 900-2000m above sea level in the Taita hills (Mukunya and Keya, 1975).

Beans are primarily grown in small scale sector, either in monocultures or mixed cropping with other crops such as maize, coffee, bananas, sorghum, millet, potatoes, cassava (Anon, 1990). In 1989, increasing adoption of line planting of two rows of beans between maize rows was noted (Anon, 1989). In the Rift Valley province, about 60% of the beans grown are intercropped with cereal crops although a few districts such as Kajiado, Kericho, Nakuru and Narok plant pure stands during the short rains (Anon,1990). In the Central province, beans are rarely grown as a pure stand as most farmers intercrop them mostly with maize (Anon, 1988).

Common varieties of *P. vulgaris* grown by small scale farmers include Mwezi Moja-GLP-1004, Rosecoco-GLP-2, Mwitemania-GLP-X.92,

Canadian Wonder-GLP-24, Red Harricot GLP-585, Zebra-GLP-806, Rosecoco-GLP-288, Rosecoco-GLP-77 and Mwezi Moja-GLP-X.1127A. (Anon, 1988). The most popular varieties are 'Canadian Wonder' grown as a late maturing cultivar mostly in Central Province; Mwezi-Moja; as an early maturing cultivar mainly grown in the drier areas such as Machakos and Kitui district; Rosecoco-GLP-2; as a medium maturing cultivar in the Western, Central and Eastern Provinces. Other land races are also grown in different areas by small scale farmers (Mukunya and Keya, 1975).

Bean yields vary greatly from place to place depending on the climate, soil conditions, seed quality, level and efficiency of insect pests and disease control and general crop management. Although yield levels range up to 3 tonnes/ha, the average yields are generally low; 750 kg/ha in monocrop and 375 kg/ha where grown as mixed stand with Maize (Njuguna, et al, 1981). Elsewhere yields of up to 5 tonnes/ha (in monoculture) and 2 tonnes/ha (in association with maize) have been obtained under experimental conditions (Roberts, 1970; Anon., 1975; Francis *et al*, 1977; Mwang'ombe *et al*, 1994).

1.2. Importance of beans

One of the major nutritional problems in many of the developing countries in the tropics is the lack of sufficient dietary proteins. A substantial part of the population in these areas, mostly low and medium income families, is not able to obtain or afford the relatively expensive and/or scarce animal protein (Roberts, 1970; Smartt, 1976; Jalil, 1977). Hence, the cultivation of food legumes in such areas remains an important nutritional aspect as they offer a cheaper alternative source of

proteins (Jalil, 1977). Beans are consumed with various other staple foods such as maize, rice, cassava, bananas and vegetables. They are eaten as immature pods, green shelled beans and also young tender leaves are used as vegetables (Goode, 1987; Purseglove, 1987). They provide a cheap source of protein, mineral salts and vitamins. Analysis of dry mature beans gives 22% protein, 1.6% fat, 57.8% carbohydrate, 4% fibres and other components for example calcium 137mg/100g (Kay and Daisy, 1979). Beans are also sold for canning and this serves as a source of income for a small percentage of Kenyans.

The whole fresh plant may be ploughed under at flowering stage and used as green manure or harvested and fed to livestock (Mukunya and Keya, 1975), although this is hardly practiced in Kenya. The dry threshed residual (straw) are used as mulch or as animal feed. Bean plants also harbour rhizobium bacteria which fixes free nitrogen from the atmosphere, thus maintaining soil fertility. However, the average farmer mainly grow beans for dry seeds and food.

1.3. Bean production problems

The low yields obtained in bean production are attributed to a number of constraints, most important of which are diseases, insect pests and weeds (Njuguna, *et al*, 1981). In Kenya, the diseases of beans considered to be of major economic importance include rust, angular leaf spot, anthracnose, charcoal rot, white mould, *Fusarium* wilt, *Ascochyta* leaf spot, bean common mosaic, haloblight and common blight of beans (Hubbeling, 1973; Mukunya and Keya, 1975; Njuguna, *et al*, 1981; Mwang'ombe *et al*, 1994). These diseases cause considerable damage on beans and the damage

caused depends on such factors as climate, altitude, cultivar grown, distribution and variation of the pathogens and whether or not the latter are seed-borne (Njuguna, *et al*, 1981; Isanda *et al*, 1993; Isanda, 1994). Due to the high cost of commercial seeds, farmers use their own seeds from the previous harvest and this implies that unhealthy seeds planted in poor soils with poor cultural practices make plants prone to pest and disease attack (Isanda *et al*, 1993; Isanda, 1994). This leads to poor yields making this a vicious cycle (Mutitu and Musyimi 1980). Bacterial and fungal diseases are generally more important in the humid tropics and subtropics, while virus diseases are usually more severe in the drier areas (Kay and Daisy, 1979). Greenburg *et al* (1987) showed that losses in bean production in Zambia could be upto 100% due to bean common mosaic virus and anthracnose, 80% due to angular leaf spot and 50% due to bacterial blight.

Angular leaf spot of beans (*Phaseolus vulgaris* L.) caused by *Phaeoisariopsis griseola* Sacc. is a common disease in Kenya. When the weather conditions are favourable, the disease can be very destructive by causing heavy defoliation (Karanja *et al*, 1994). The pathogen also invades pods and stems and the fact that angular leaf-spot can cause economic losses, especially where ideal conditions for its multiplication prevail cannot be underestimated.

1.4. Control of Angular leafspot

The recommended methods for the control of ALS include use of chemicals (Taylor and Dudley, 1977), use of clean seeds, crop rotation and removal of previously infected crop debris (Cardona-Alvarez, 1956; Zaumeyer and Thomas, 1957; Barros *et al*, 1958;

Grogan and Kimble, 1967). However, the effectiveness of these methods are limited due to the high production costs of using chemical control, the ability of the pathogen to survive in plant debris for a long period of time, land unavailability to practice crop rotation and the fact that small scale farmers in Kenya tend to use their own seeds from the previous season (Isanda, 1994; Karanja *et al*, 1994).

For the farmer, the use of resistant varieties combined with other pest control practices is regarded to be an effective and relatively cheap means of controlling the disease. Breeding programs are thus an important strategy for the control of this disease. However, for breeding programs to succeed, the extent of pathogenic variation existing within the pathogen population must be determined and well understood. This can be done through race surveys and virulence analyses. Disease screening then can be based on a wide-based pathogenic spectrum in order to obtain relatively stable resistance. Likewise, establishing the mechanism of resistance in bean varieties to angular leafspot is of paramount importance.

Much work has been done and documented on bean anthracnose, rust, bacterial blights, viruses and many other bean diseases resulting in improved methods of control of these diseases (Mulindwa, 1980). *Colletotricum lindemuthianum, Uromyces appendiculatus,* Bean common mosaic virus and *Pseudomonas syringae* pv *phaseolicola* are known to exist in different physiological races (Barrus, 1911; Harter and Zaumeyer, 1941; Dean and Wilson, 1959; Kinyua and Mukunya, 1981;). However as for *P. griseola,* only limited studies on physiological specialization have been conducted in the past (Villegas, 1954; Silvera, 1967; Alvarez-

Ayala and Schwartz, 1979; Buruchara, 1983) but no pathogenic variation studies have been carried out on the Kenyan isolates to confirm the same. Thus the aim of this project was:

(i) To determine if *P. griseola* Sacc. exists in different pathogenic forms (races) in important bean growing districts in Kenya.

(ii) To determine the range of symptoms induced by *P. griseola* on *P. vulgaris* cultivars.

(iii) To study colonization of susceptible and resistant tissues of advanced lines of *Phaseolus vulgaris* L. developed at Kabete Campus.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Nomenclature of beans.

The common bean (*Phaseolus vulgaris* L.) is also known as French bean, kidney bean, haricot bean, salad bean, string bean or frijoles in different areas. It is classified under the division Spermatophyta, subdivision Angiospermae, class Dicotylidonae, subclass Rosidae, order Fabales, family Fabaceae or Papilionaceae and genus *Phaseolus* (Holmes,1986). The genus *Phaseolus* includes about 31 species of which about 10 are important cultivated crops. *Phaseolus vulgaris* is the best known and most widely cultivated species of genus *Phaseolus* (Allen, 1983). They are annuals or perennials, twinning or bushy herbs with large trifoliolate leaves, stipules and stipels, typically papilionaous flowers in axillary or terminal racemes (Purseglove, 1987).

2.2. Geographical distribution of Angular leafspot

Angular leafspot of beans has such a characteristic appearance on foliage and the fungus fruiting structures are so distinctive that the disease was noted and described as early as 1878 in Italy (Saccardo, 1886). Since then, it has been reported worldwide where beans are grown and is especially prevalent and destructive in tropical and subtropical countries. It is considered a major problem in many of the bean growing regions (Zaumeyer and Thomas, 1957; Zaumeyer 1968; Gutierrez *et al*, 1975; Graham, 1978). It has been reported in the United States, Australia, Japan, India, Iran, Israel, Europe and Africa.

Occurence of this disease is often sporadic and when environmental conditions are favourable, infection can reach epidemic levels. It is common in South American countries (Zaumeyer and Thomas 1957) and widespread in Africa (Allen, 1983). In U.S.A., the disease occurs often in the Southern tropical and subtropical states but in the northern more temperate areas, its occurence is sporadic (Cole, 1966). In Sierra Leone, it was first recorded by Deighton in 1952. Hendricks (1940) first observed the disease in Congo at Mulungu station in 1938, while Wallace (1952) noted how it spread in Tanzania. In Uganda, the disease was first recorded by Hansford in 1938. In Kenya, angular leaf spot is known to occur in all the major bean growing areas (Njuguna, *et al*, 1981).

2.3. Economic importance of angular leafspot.

When conditions for its development are favourable, angular leaf spot can cause extensive damage. In 1955, the disease was first recorded on local varieties in Varacruz area of Mexico although by then, it was also noted as being very severe in the southern countries of central America and Columbia (Yerkes and Crispian, 1957). In 1954, an epidemic causing 50% loss was recorded in Wisconsin (Cardona-Alvarez and Walker, 1956). Tanzania had its most severe attack in 1947 due to prolonged rains resulting in a long period of cool humid conditions for the fungus to spread and multiply (Wallace, 1952). Prior to 1953, the disease was regarded as being of minor importance on beans in New Caledonia (Barros, *et al*, 1958). It became very important when it caused heavy losses in dry bean crops especially in Valle de cauca (Barros, *et al*, 1958).

In Pennyslvania in 1966, Cole Junior reported that angular leafspot caused 10-50% reduction in yields with a high incidence of small shrivelled beans (Cole,1966). In the following year, severe foliage spotting, defoliation and death occurred at the pod filling period. Although no quantitative comparisons were made, visual observation indicated that lesions and consequent defoliation were primarily responsible for reduction of bean size and yield. Defoliation mainly occurs starting from the flowering stage and beginning of pod filling stage. Due to reduced foliage, the little food manufactured by the plant would be directed towards the up-keep of the plant rather than pod development and filling. In Ethiopia losses of 50-60% due to this disease were realised (Galatto and Meossi, 1973), while in Argentina in 1974, Fortugno, (1974) recorded 30-40% loss in bean yields due to the disease. In Colombia, the disease reduced dry bean yields by 80% (Schwartz, *et al*, 1979). In Kenya, the disease is considered to be economically important and causes considerable damage on beans (Anon, 1989). However, no data is available on the actual yield or potential loss due to the disease, or the feasibility of specific strategies to control this disease.

2.4. ETIOLOGY

2.4.1. Nomenclature of *P. griseola*.

The angular leafspot fungus *Phaeoisariopsis griseola* Sacc on *Phaseolus vulgaris* L. was described for the first time by Saccardo (1878) in Italy as *Isariopsis griseola*. Later, the same organism was described under the names *Graphium laxum* Ell, (Ellis, 1881), *Cercospora columnarea* Ell, and Ev. (Ellis and Everhart, 1893), *Phaeoisariopsis griseola* (Sacc.) Ferr.

(Ferraris, 1909) and *Lindaumyces griseola* Gonz-Frag. (Gonzalez-Fragoso 1927). However, after a critical review of these descriptions, Harter and Zaumeyer (1944) came to a conclusion that all the later names were synonymous with *Isariopsis griseola* Sacc. Henceforth, the generic name *Phaeoisariopsis griseola* (Sacc.) Ferr is used as the causal organism of angular leafspot of beans. Information collected by Zaumeyer and Thomas (1957), place *P. griseola* (Sacc.) Ferr in the family *Dematiaceae* of the subdivision Deuteoromycotina, class Hyphomycetes.

2.4.2. Morphological characteristics of *P. griseola*

P. griseola Sacc. produces clusters of parallel columnal conidiophores known as synnemata. The number of conidiophores to a synnema and size of synnema show considerable variation. Miles (1917) observed that the synnemal conidiophores varied in number ranging from 8-40. Various workers have also reported varying sizes of the synnema, ranging between 94-680µ in length and 20-70µ in width (Saccardo, 1886; Miles, 1917; Srinivasan, 1953; Zaumeyer and Thomas, 1957; Hocking, 1967; Ellis, 1971). Their sizes have also been noted to show variation. The length and the width of the conidia are reported to range between 20-80µ and 3.4-8.8µ respectively (Saccardo, 1886; Benlloch 1944; Zaumeyer and Thomas, 1957; Llanos, 1957; Hocking, 1967; Ellis, 1971). Zaumeyer and Thomas (1957) observed that *P. griseola* had conidia with one to three septa and rarely four. However Benlloch (1944), Hocking (1967) and Ellis (1971) found conidia which had upto six septa. Llanos (1957) observed that conidia with three and four septa were more common, but those with one and five septa were rare and it was very difficult to find a conidium without a

septum. Buruchara (1983) reported that conidia of *P. griseola* were 38.5µ in length and 6.4µ in width. Karanja *et al* (1994) observed that most conidia had 1 to 5 septa and were 44µ long by 13.9µ wide. However, there is no evidence to suggest that some relationship exists between these morphological variations and any physiological or pathogenic characteristics of the fungi.

2.4.3. Host range of *P. griseola*.

Angular leafspot caused by *P. griseola* attacks only common beans (*Phaseolus vulgaris* L.) and lima beans (*Phaseolus lunatus* L.). During the growing seasons of 1951 to 1954 at Medollin Colombia, single rows of susceptible beans were planted alternatively with double rows of other legume species including *Lablab purpureus, Cajanus* spp. *Crotalaria* spp and *Soja max* (L.) Piper but infection only occurred on *P. vulgaris* and *Phaseolus lunatus* (Cardona-Alvalez and Walker, 1956).

2.4.4. Infection by *P. griseola*.

The pathogen penetrates into the host tissue through the stomata and progresses intercellularly through the mesophyll cells and the palisade layer. As necrosis of affected cells sets in, the fungus becomes intracellular and extends rapidly until it becomes restricted by vascular bundles. It grows in a temperature range of 8-28°C with an optimum temperature of 24°C. (Cardona-Alvarez and Walker, 1956). Coremial formation occurs only in humid atmosphere and is completed about 24 hours at 24°C but heavy sporulation occurs after 72 hours or more.

Once penetration has occurred, disease development can proceed in relatively dry atmosphere and stromata is formed abundantly in substomatal cavities. Once spores are formed, low humidity is favourable to spore release from coremia and to dissemination. Climatic factors that promote disease development are thus moderate temperatures from 16°C to 28° with an optimum of 24°C and rainy or humid periods for 24 to 48 hours alternated with dry windy periods.

2.4.5. Symptomatology of *P. griseola*.

Symptoms induced by *P. griseola* on beans develop on leaves, stems and pods. Lesions can appear on primary leaves within 6 days after inoculation, but usually do not become prevalent until the late flowering or early pod-set stage. The spots are small, angular, dark brown and often so numerous that they give the foliage a checkerboard appearance. Lesions may increase in size, coalesce and cause necrosis and yellowing.

When *P. griseola* attacks a plant, spots originate on the underside of the leaf and are delimited by the veins and veinlets. At first, the lesions are grey, later turn brown and attain an angular shape because of limitation by veins. Pod lesions are roughly circular and reddish brown with dark brown borders. Stem lesions are dark brown and elongate. On all lesions, dark stroma appear in abundance; in moist weather, macroscopically visible coremia are formed on the stromata. The spots appear first on the lower foliage and gradually increase as the season progresses. At mid-season, all plants in severely

affected parts of the field maybe dead. Infected plants pass through a usual sequence of spotting, necrosis, chlorosis and defoliation.

The fruiting fungus produce a grey mold on the lower surface of the leaf and may cover the pods and stems. Gremmens (1947) reported that infected Beka brown beans showed quadrangular dark brown spots. He also gave microscopic appearance of underside of leaf which showed masses of dark brown coremia bearing elongate often slightly curved, brown to quadricellular conidia.

2.4.6. Variability in *P. griseola*

The mechanisms which bring about variations in pathogens include mutation (somatic), recombination in the sexual state, heterokaryosis, parasexualism and cytoplasmic adaptation. Since the perfect state of *P. griseola* has not been found to occur naturally, any cultural, morphological or pathogenic variations observed are probably due to the genetical changes brought about by either one or more of the mechanisms of mutation, heterokaryosis or parasexualism (Buruchara,1983).

The presence or absence of different strains of *P. griseola* is an important area which many research workers have tended to overlook. If different strains are present but not identified, confusing and conflicting results can be obtained while dealing with other aspects of the fungus especially during screening and breeding for resistance. Some investigators in the past have given indications that *P. griseola* exhibits pathogenic variation. For example, Wallace (1952) in Tanzania demonstrated the existence of more than one strain but his work was not confirmed by any follow up experiments. Diaz *et al* (1965) noted that

morphological and epidemiological data in Lake Valencia basin suggested the existence of different races but again, there was no follow-up experiments on this observation. In 1966, Hocking noted a new virulent form of *P. griseola* causing circular leaf spot on french beans in a farm in Arusha.

Villegas (1954) was able to group 33 single spore isolates of *P. griseola* from Colombia into 13 races on the basis of their differential reactions with 11 bean lines but doubted the genetic purity and uniformity of his lines. Using young seedlings, he found that only 2 of the 14 lines showed resistance to most of the isolates but none was immune to all isolates. Preliminary studies by Alvarez-Ayala and Schwartz (1979) at CIAT using 5 isolates from Columbia and 5 cultivars also suggested that pathogenic variation in *P. griseola* exists. Buruchara (1983) using 20 isolates from Columbia and one isolate from Wisconsin U.S.A. managed to group them into 7 pathotypes based on their reactions on 6 bean cultivars. Leach *et al* (1939) pointed out that if pathogenic races exist in different areas, one variety resistant in one area maybe found susceptible in another.

Indication of field occurence of physiological races of plant pathogens are generally based on 2 criteria:

(a) Gradual breakdown of disease resistance in a variety in a given area and

(b) The large differences in varietal reactions at different locations or countries (Chiu *et al* 1965).

Demonstration of occurrence of physiological races in a fungus is usually based on various types of host reactions arising from various host pathogen interractions. The host reactions, either qualitative or quantitative are quantified in either specific or

arbitrary units which are usually categorized as either resistant or susceptible. They include reactions such as:-

(a) susceptible or resistant.

(b) infection types.

(c) percentage of infected plants (Armstrong and Armstrong, 1981).

(d) percentage of leaf area covered with lesions.

(e) sporulation capacities of different races of a pathogen in a given host (Nelson, 1973).

The host pathogen interactions which result in host reactions a,b,c and d measure host physiology whereas e relates mainly to the epidemiology capabilities of the pathogen.

Physiological races are determined by use of differential hosts. Systematic designation of the tested isolates into races should be based on known resistant genes or genetic compositions of differential cultivars after analysing them genetically (Drijfhout, 1978). Use of differential cultivars of unknown genome may give results not comparable to those obtained in other areas, especially if the pathogen population is constituted of pathotypes different from those occurring at the source of the differentials. To avoid discrepancies in results, co-operative programs between interested countries have to be made to develop regional and/or international set of differentials. This would entail development, multiplication and distribution of seeds of differentials of known genomes.

2.4.7. Control of Angular leaf spot

The recommended methods for the control of angular leafspot can be grouped into cultural, chemical and use of resistant varieties.

2.4.7.1. Cultural control.

As the disease is believed to spread from infected straw and volunteer plants growing out of season, Barros *et al* (1958) suggested crop rotation and restricting sowing to one time of the year. Wallace (1952) suggested that on neighbouring farms, sowing should not spread over a long period and debris from the previous season should be destroyed. Use of clean seeds is also an effective method of control since the pathogen is also seed borne (Grogan and Kimble, 1967; Karanja, 1994).

2.4.7.2. Chemical control

Among the chemicals used, Zineb (Zinc Ethylene 1,2 - bisdithiocarbamate) appears to be more effective. Barros *et al* (1958) found that spraying 250g/L (1137.5 litres) of Zineb per hectare 5-6 times was effective. In Mauritius and Malawi, it was found that 2.5 g/L could reduce the size of the spots considerably (Anon, 1959). Milatovic (1959) explained that if seeds were dusted with Ceresan (Ethylmercury chlorate), germisan, agrosan (Phenylmercury acetate) and copper carbamate at 0.1 - 0.2%, the disease would be reduced but this would affect germination also. Wallace (1952) and Oxenham (1957) used bordeux mixture (copper sulphate + calcium hydroxide) and sulphur dust and found them to be effective. Other chemicals which have been tried in Colombia by Fortugno (1974) include benzimidazole 60% at

50 g/L, benomyl (Methyl 1-butylcarbamoyl benzimidazol-1-ylcarbamate) 50 at 40g/L or triforine (1,4-di-(2,2,2-trichloro-1-formamidoethyl)-piperazine) 20% at 200ml/ha spraying 3 times in each case.

2.4.7.3. Breeding for resistance and use of resistant varieties

The effectiveness of cultural and chemical control methods is limited due to high production costs of using chemical control, the ability of the pathogen to survive in plant debris for a long period of time and land unavailability to practice crop rotation. On the contrary the development and introduction of resistant bean varieties combined with other control practices, is regarded to be an effective and relatively cheap means of reducing disease incidence and consequently reducing yield losses at the farm level.

Whereas the use of resistant varieties is the cheapest and easiest means of disease control at the farm level, it is an area where little work has been done with regard to Angular leaf spot of beans. One way of achieving this goal involves the testing of the available germplasm and development of improved cultivars with multiple disease resistance (Anon, 1979). Brock (1951) screened 164 lines for resistance to angular leafspot; 11 lines were highly resistant and showed no lesions or defoliation on the young leaves and very few on the first. Olave (1960) screened some lines for resistance to *P. griseola*; 3 lines were highly resistant, 5 moderataly resistant, 2 susceptible and 2 highly susceptible. Although some workers have tested for resistance and some have tried to incoporate it in their varieties only Barros

et al (1957) and Santos-Filho *et al* (1978) appear to have tried to find out the inheritance of this resistance. Santos *et al* (1978) used 44 lines to test the resistance to *P. griseola*. Two lines were resistant and one very resistant. Resistance to the most pathogenic local isolate proved to be recessive and controlled by 2 or 3 independent factors. Mulindwa (1980) reported that resistance to angular leafspot is governed by a single dominant gene.

Resistance to *P. griseola* has been shown to be of the race specific type where a cultivar can resist infection by some of the isolates but not all (Mulindwa, 1980). Studies made in the past regarding inheritance of resistance to angular leafspot, showed that resistance in the bean (*Phaseolus vulgaris* L.) cultivars `Caraota 260' `Decal', `Maravilla' and `Line 258' as well as *Phaseolus coccineus* L. cultivar PLB 257, were governed by either one or two to three independent factors (Barros *et al;* 1957; Cardona-Alvarez, 1958; Santos-Filho *et al*. 1976; Singh and Saini, 1980) which is characteristic of race-specific inheritance (Van der Plank, 1968). This type of resistance is often complete (Eenink, 1976) but its level maybe influenced by genetic dosage effect (Dunn and Mamm, 1970), modifier genes (Rousele, 1974) or the physiological age of the plant (Bartos *et al*., 1969). Extraneous factors such as soil temperature, air humidity or light intensities also appear to raise or lower the level of this monogenic resistance (Hubbeling, 1966; Walker and Williams, 1973). The presence of a resistant reaction corresponding to disease severity index 2 rather than an immune reaction is probably due to the effect of any one or more of the above factors which influence the level of monogenic resistance.

There are indications that rate-reducing resistance against *P. griseola* occurs in some of the bean cultivars whereby cultivars show differences in the time when symptoms appear and the extent of disease severity (Buruchara, 1983). The appearance of symptoms two or three days later in the resistant varieties than in the susceptible ones maybe attributed to either long latent periods or race-specific resistance in the cultivars (Van der Plank, 1968; Parlevliet 1979,). Whether or not this resistance is race specific is not yet known. Van der Plank (1968), considered this apparent infection rate-reducing resistance to be of the race specific type. However Toriyama (1975), Ou (1979) and Parlevliet (1979), reckoned that this type of resistance which is incomplete can also be race specific. Reduction of the rate of infection reduces the epidemic development of the disease by decreasing the reproduction rate of the pathogen (Parlevliet, 1979). The factors of resistance which lead to the latter are the reduction in infection frequency or lesion numbers, lengthening of the latent period and the decrease in sporulation capacity. These aspects need to be considered and incorporated in future studies when attempting to develop stable resistance against *P. griseola* in beans. Race non-specific type of resistance is more durable because the pathogen finds it more difficult to adapt to the several loci involved whereas with race-specific resistance, the pathogen population tends to adapt to the host more easily and results in 'loss' of resistance (Parlevliet, 1978).

For breeding programs to succeed, the extent of pathogenic variation existing within the pathogen population must be determined (Zaumeyer 1966 and Williams, 1979) by race surveys and

virulence analyses. Use of resistant varieties is handicapped by the new races that keep developing thus overcoming the host resistance which is race specific. It is therefore of paramount importance to breed for race non-specific resistance which is more durable.

In the past, bean cultivars that have been reported to have resistance against *P. griseola* include; 'Kentucky Wonder' (Gardener and Mains, 1930). 'Alabama No. 1, 'California small white No 643', Epicure (Brock, 1951), 'Cauca -27A' (Anon, 1956; Olave, 1958); 'Caraota-260' (Santos-Filho *et al* 1976) and 'G 4421' (Avila, 1979). However Alvarez-Ayala and Schwartz (1979) observed that cultivars 'Alabama No 1', Cauca 27A and 'Caroata-260' were susceptible to 1, 1 and 3 isolates respectively out of the 5 isolates they used.

Some of the cultivars previously reported to be good sources of resistance to angular leaf spot in various countries have been found to be susceptible in either the same or different countries. In Columbia, all the above 7 cultivars were found to be susceptible to at least one or more of the 20 Colombia and one USA isolate of *P. griseola* tested (Buruchara, 1983). However some of the cultivars were resistant (immune) to some of the isolates, implying that there exists a host-pathogen specialization relationship. In Brazil, cultivars 'Mantaigao Proto 20' and Cauca -1680-N' were initially reported to have high degrees of resistance (Vieira, 1974). Santos -Filho *et al* (1976) found the two cultivars to be highly susceptible. They nevertheless observed that 'Caraota 260' was highly resistant to a mixture of 10 local isolates.

Resistance can be described as an inherent ability of the host plant to resist or restrict establishment and subsequent activities of a potential pathogen. The host can either resist establishment of a successful parasitic relationship by restricting infection site and infection process or it can resist colonization and growth of the pathogen subsequent to a successful infection. The former is referred to as hypersensitive reaction, specific resistance, vertical resistance or major gene resistance, while the latter is referred to as field resistance, partial resistance, horizontal resistance, polyygenic or minor gene resistance.

The mechanism of resistance can either be mechanical or chemical, both of which are either pre-existing or responsive. The pre-existing structural defences include the primary cell wall strengthened by cutin, suberin, lignin, silicon waxes or calcium. Induced mechanical barriers or post infectional structural defences include:-

(i) Alteration of existing cell walls by lignification, accumulation of calcium, silicon, quinones and melanins.
(ii) Deposition of new wall like material referred to as papilla.
(iii) Barrier formation by renewed cell division.
(iv) Slow lesion formation and slow sporulation.
(v) Cork barrier formation.
(vi) Vascular occlusion.
(vii) Hypersensitive reaction (Kiraly *et al*, 1972).

Likewise, in chemical resistance, some chemicals are constitutive in the plant while others are induced, for instance phytoalexins

and glycoproteins. In a resistant plant, one or both resistance mechanisms are in operation and resistance can occur at any level or stage of disease development process for example at germination, penetration, colonization or sporulation.

The structural and biochemical events leading to accommondation or rejection of a fungus by a potential host are likely to be unique for each gene-for-gene interaction as has been amply demonstrated by the work of Ellingboe (1975) using various races of *Erysiphe graminis* and various genotypes of its hosts (wheat and barley) and the studies of Heath (1974) on cowpea rust. However rapid collapse and death of one or more host cells at the penetration site, linked with the death or cessation of growth of the invading fungus appears to accompany resistance in a wide range of gene-for-gene interactions. In some cases, rapid death of host cells maybe an integral part of the resistance process while in others, it maybe a consequence of resistance only, thus generalisations based on macroscopic observations maybe very misleading

As long ago as 1892, Cobb proposed a mechanical theory of rust resistance which stated that morphological characters such as a thick cuticle, a waxy covering, small stomata, a large number of leaf hairs and upright leaves might be responsible for the resistance of some wheat varieties to *Puccinia graminis*. Fungal colonization of susceptible tissues can be localized in discrete areas by barrier tissues which are resistant to invasion. These usually comprise suberized or lignified cells and may vary in extent between varieties, plant organs or with age. Hursh (1924) noted that mycelium of *P. graminis* grew only in the chlorenchyma of wheat stems which in some varieties was very

broad and confluent but in others was fragmented in small bundles by sclerenchyma which the fungus cannot colonise. Such limitation of the pathogen growth reduce damage to the host and also limit sporulation. It is conceivable that a thick and tough epidermis may restrict the fructification of a pathogen or limit its final development and effectiveness as secondary inoculum. Hafiz (1952) reported that the glandular hair population on stems and leaves of resistant gram types was greater than that on susceptible types. Since the glandular hairs are known to secrete malic acid which is known to inhibit spore germination and retard hyphal growth, this differential in hair population may in part account for the differences in susceptibility and resistance in gram to *Mycosphaerella* blight.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Determination of pathogenic variability in isolates of *Phaeoisariopsis griseola* Sacc.

3.1.1. Sources of bean tissue infected by *P. griseola*.

Isolates of *P. griseola* used in this study were obtained from naturally infected bean (*Phaseolus vulgaris* L.) samples collected from farmers fields and Research centres experimental fields in fifteen bean growing districts in Kenya, representing varying ecological conditions (Table 1). The survey was undertaken during the short rainy season of 1993 and long rainy season of 1994. The fields were selected randomly and bean plants examined for angular leafspot symptoms on leaves, pods and stems. The diseased bean specimens were collected in paperbags which were clearly labelled. The specimens were preserved in the refrigerator at 4°C prior to isolation. Seven isolates were obtained from Central Province (Murang'a, Kiambu, Thika, Nyeri, Kirinyaga and Nyandarua), two from Coast Province (Taita Taveta), three from Eastern (Embu, Meru and Machakos), two from the Rift Valley (Nakuru and Kajiado), one from Nyanza (Kisii), one from Western (Vihiga) and two from Nairobi (Embakasi and Kabete). A total of 18 isolates were used.

3.1.2 Isolation of *P. griseola*

Isolations were made from lesions of naturally infected bean leaf tissues showing fungal sporulation. In case of non-sporulating lesions, the fungus was induced to sporulate by incubating the infected tissues.

Table 1: SOURCES OF *PHAEOISARIOPSIS GRISEOLA* SACC. ISOLATES.

Area of origin			Climatological conditions		
District	Place of collection	Isolate	Altitude (m)	Mean annual rainfall (mm/yr)	Mean annual temperature (°c)
Murang'a	Kangema	PG1	2450	1525-1780	12-16
Kiambu	Ndeiya	PG2	2070	760-1015	18-22
Thika	KARI	PG3	1554	510-760	16-20
Nyeri	Wambugu farm	PG4	1750	1015-1270	16-22
Nyeri	Mweiga	PG5	1730	760-1015	16-20
Kirinyaga	Kerugoya	PG6	1650	1270-1525	16-20
Nyandarua	Ndaragwa	PG7	2280	510-760	18-22
Taita Taveta	Josa	PG8	1510	1015-1270	22-26
Taita Taveta	Werugа	PG9	1820	1015-1270	22-26
Embu	KARI	PG10	2100	760-1015	16-20
Machakos	Katumani	PG11	1500	510-760	20-22
Meru	Chuka	PG12	2300	1525-1780	12-16
Nakuru	Bahati	PG13	2195	1015-1270	14-18
Kajiado	Ngong	PG14	1800	760-1015	22-26
Kisii	Keumbo	PG15	1700	1525-1780	18-22
Viniga	Sabatia	PG16	1650	1525-1780	20-24
Nairobi	Embakasi	PG17	1650	510-760	16-20
Nairobi	Kabete	PG18	1810	760-1015	18-22

3.1.2.1. Incubation of infected tissue

Small pieces of infected tissues were surface sterilized with 10% (w/v) sodium hypochlorite for 5 minutes and rinsed in 5 changes of sterile distilled water. They were then placed in moist chambers prepared using filter paper and glass boxes. The surface-sterilized, infected leaf parts were incubated as described by Karanja *et al* (1994).

3.1.2.2. Preparation of Bean leaf dextrose agar and water agar plates

Bean leaf dextrose agar medium (BLDA) prepared as described by Karanja *et al* (1994) was used in the isolation of the pathogen. 100g of freshly collected bean leaves were weighed and crushed in a blender with a small amount of distilled water. The mixture was filtered through a double layer of cheese cloth and sterile distilled water added to make to 1 litre. 20g of glucose and 20g of agar were then added and the pH of the mixture adjusted to 6.8. The mixture was sterilized in an autoclave at 121°C at 15 psi for 15 minutes and approximately 20 ml were dispensed into sterile petri dishes.

Water agar plates were prepared by adding 20g of agar to 1 litre of water and the same sterilizing and dispensing procedure as for BLDA followed.

3.1.2.3. Inoculation of water agar plates.

A porcelain tile was swabbed with 70% alcohol and laid on a bench surface. A sterilized glass slide was laid on the tile and a few drops of sterile distilled water placed at the centre of the slide. Infected material showing fungal sporulation was

placed on the stage of an ERMA binocular dissecting microscope and conidia on the synnemata were touched with the tip of a fine moistened mounted needle without touching the host material. The conidia collected were transferred on to the water droplet on the slide, and stirred with a wire-loop to form a spore suspension. A film of the spore suspension was captured by withdrawing the wire-loop after stirring and streaked across the surface of water agar plates, using four strokes to distribute the spores. The plates were incubated at 19-21°C and conidia germination monitored using a compound microscope.

3.1.2.4. Single spore isolation of *P. griseola*.

Bean leaf dextrose agar (BLDA) was used to culture *P. griseola* isolates. After two days of incubating water agar plates inoculated with *P. griseola*, a small block of the medium containing a single germinating conidia was cut out, transfered on to BLDA plates and incubated at 21-24°C in darkness for 14 days (Alvarez-Ayala and Schwartz, 1979). The resulting single spore cultures were separately bulked further using BLDA while maintaining their identity.

3.1.3. Pathogenicity test

3.1.3.1. Bean plants.

Bean (*Phaseolus vulgaris* L.) seeds of cvs Rosecoco-GLP-2 and M29 were surface sterilized using 10% sodium hypochlorite and rinsed using 5 changes of sterile distilled water. The seeds were sown in polythene bags containing steam sterilized soil composed of soil, manure, sand and ballast in the ratio of 2:1:1:1

respectively. Seedlings were allowed to grow in the glasshouse until the first trifoliolate leaves were fully formed.

3.1.3.2. Inoculum preparation.

Plates with 14 day-old cultures of *P. griseola* isolates were flooded with sterile distilled water and the inocula prepared as described by Karanja *et al* (1994). A bent sterile glass rod was used to scrap off the conidia from the culture surface. The suspension obtained was filtered through a double layer of cheese cloth and its concentration determined by use of a Neubauer improved haemocytometer. The conidial concentration was then adjusted to $2x10^6$ conidia ml^{-1}.

3.1.3.3. Inoculation and incubation of bean plants.

Three week old bean seedlings of cvs Rosecoco-GLP-2 and M29 were covered with transparent polythene bags 24 hours before inoculation. Using a half litre Baygon atomizer (Bayer E.A.),the plants were inoculated mainly on the abaxial side of the first trifoliolate and primary simple leaves at a distance of 10-15 cm until runoff. Control plants were sprayed with sterile distilled water. Polythene bags were returned and then removed 24 hours later. The plants were incubated on a greenhouse bench and examined daily for angular leafspot symptoms development for upto 21 days. To fulfil Koch's postulates, *P. griseola* was reisolated from infected leaf tissue.

3.1.4. Maintenance of *P. griseola* cultures

Pure cultures of *P. griseola* isolates were subcultured on BLDA slants and incubated at 24°c in the dark for 10 days. Thereafter, the slants were maintained at 4°c.

3.1.5. Slide cultures of *P. griseola* isolates.

Slide cultures of ten *P. griseola* isolates (PG1, PG2, PG4, PG5, PG7, PG8, PG10, PG11, PG12 and PG15) were prepared for further identification of the fungus. A modification of the slide culture technique described by Riddell (1950) was used for further studies of the morphological characteristics of the fungus. A bent glass rod was placed on a filter paper-lined bottom of a petri-dish and a clean microscope slide placed on top of it. The set-up was sterilized in an autoclave at 180°c for one and a half hours. One cm^2 agar blocks were cut out of sterilized BLDA medium plates prepared as in section 3.1.2.2. One agar block was placed on the sterile microscope slide and seeded at the centre with conidia of *P. griseola* using a sterile inoculating needle. A sterile cover slip was centrally placed on the agar block and the set-up incubated at room temperature (20-24°c) in the dark. To maintain high humidity within the petri-dish, the filter paper was kept moist by periodically adding 2% aqueous solution of glycerine. On the 10th day after incubation, the cover slip was lifted gently and the agar block discarded. The cover slip and the microscope slide were separately mounted in a drop of lactophenol in cotton blue. The slides were examined under an SM-LUX compound microscope to determine the colour of

the conidia, nature of conidia attachment, hyphal fusion, e.t.c. and the observations noted.

3.1.6. Determination of variation in conidial size and growth of the isolates on artificial medium.

3.1.6.1. Conidial size.

A total of ten isolates used in section 3.1.5. were used to determine the variation in conidial size. Single spore cultures of the isolates were grown in petri dishes containing bean leaf dextrose agar for 14 days in a dark incubator set at 24°C. On to each culture plate, 5 ml of sterile distilled water was added and the culture surface gently scrapped using a bent glass rod to make a spore suspension. One hundred conidia per isolate were measured to determine the length, width and number of septa using an SM-LUX compound microscope fitted with a micrometer (Graticule). The width was determined by measuring the widest section of the conidia. A completly randomized design with 4 replicates per treatment (isolate) was used.

3.1.6.2. Growth variation.

The ten isolates used in section 3.1.6. were cultured to determine variation in hyphal growth. Conidia suspension were prepared as described in section 3.1.3.2. and their concentration determined using a haemocytometer. The concentration was adjusted to 2×10^{3} conidia ml^{-1} and ten drops of the suspension plated on petri dishes containing about 20 ml of BLDA medium. The plates were incubated at 24°C in the dark and observed after 10 days. A completely randomised design with three replicates was used.

3.1.7. Pathogenic variability among *P. griseola* isolates

3.1.7.1. Sources of *Phaseolus vulgaris* cultivars.

A total of 30 bean cultivars were used. A set of 22 differential varieties for *Phaeoisariopsis griseola* were obtained from CIAT, Colombia. Six bean lines developed at Kabete Campus, University of Nairobi (some susceptible and others resistant to angular leafspot) and two of the common bean cultivars grown in Kenya were also used. The six bean lines developed at Kabete Campus were selected on the basis of their reaction to natural inoculum of *P. griseola* in the field.

The CIAT differentials used included BAT76, Calima, Montcalm, A62, BAT332, G5686, Caraota, Seafarer, Alabama No.1, G9603, BAT1647, A21, P.Checha, A235, G1805, A212, Amendoin, G2858, A301, A339, C49242 and A285 while the six lines from the Bean Breeding Program at Kabete Campus included M14, M16, M20, M26, M29 and E2. The two locally grown bean cultivars used in this study were Rosecoco-GLP-2 and Mwitemania-GLP-X.92.

3.1.7.2. Seed potting.

Seeds were sown as described under section 3.1.3.1. In each pot 3 seeds of the cv/line were sown each representing a replicate. A completely randomized design with 3 replicates was used. For confirmation, the experiments were repeated once.

3.1.7.3. Inoculum preparation, inoculation and incubation of bean plants.

Inoculum for each isolate was prepared as described under section 3.1.3.2. and the concentration adjusted to $2x10^{7}$ conidia ml^{-1}. Three week old bean seedlings were used. Prior to inoculation, the plants were thoroughly watered and covered with polythene bags for 24 hours to increase the humidity. Inoculations were made outside the greenhouse and each isolate was handled separately. The inoculum was sprayed onto both surfaces of the first trifoliolate and primary simple leaves using a half litre Baygon atomiser until runoff. The plants were then covered with polythene bags for 24 hours after which they were uncovered and incubated on a bench in the greenhouse. The whole experiment was laid down as a completely randomised desigh presented as a 30x18 factorial.

3.1.7.4. Angular leaf spot assessment.

Angular leafspot assessment was made 14 and 21 days after inoculation and was based on a CIAT angular leafspot evaluation scale of 1 to 9 (CIAT, 1992).

1 = No visible symptoms of the disease.

3 = Presence of few small nonsporulating lesions covering approximately 2% of the leaf area.

5 = Presence of several generally small lesions with limited sporulation covering approximately 5% of the leaf area.

7 = Abundant and generally large sporulating lesions covering approximately 10% of the leaf area. On the foliage the lesions may coalesce producing larger infected areas associated with chlorotic tissue. Lesions are also generally

present on the stem and branches.

9 = 25% or more of leaf area is covered by large sporulating and often coalescing lesions. Leaf tissues are generally chlorotic resulting in severe and premature defoliation. Abundant sporulating lesions are present on stem and branches.

Cultivars were scored as susceptible corresponding to the symptoms indexed as 5 to 9 or as resistant corresponding to index 1 to 3. The disease severity percentages obtained were transformed using the arcsin transformation and analysed statistically.

Standard area diagrams representing the above scale were used as a guide to estimate disease severity (Van Shcoonhoven and Marcial, 1987). In addition, incubation periods (days from inoculation to the time the lesions were first observed) and the different type of symptoms of angular leafspot on the leaves were also evaluated.

3.2. Bean tissue colonization by *Phaeoisariopsis griseola*

3.2.1. Bean plants.

Two bean line, a resistant one (line M26) and intermediately resistant (line M29) to angular leafspot obtained from the Bean Breeding Programme at Kabete campus and cv Rosecoco-GLP-2 (known to be highly susceptible to ALS under field conditions) were used. Seeds were potted as described in section 3.1.3.1. and the seedlings allowed to grow in the greenhouse until they were three weeks old.

3.2.2. Inoculum preparation, inoculation and incubation of bean plants.

P. griseola isolate PG18 obtained from Kabete campus field station was used. The inoculum was prepared as in section 3.1.3.2 and prior to inoculation, the plants were covered with transparent polythene bags for 24 hours. Inoculations were carried out in marked circles on both sides of the first and second trifoliolate leaves. Control plants were sprayed with sterile distilled water. The polythene bags were returned, then removed after 24 hours and the plants incubated on a greenhouse bench.

3.2.3. Light microscopy.

3.2.3.1. Preparation of clearing and staining solution

The clearing and staining solution was prepared by successively adding the following compounds in the following proportions (for approximately 1 litre of solution) and mixing them with a magnetic stirrer until all solids were dissolved (Bruzzese and Hasan, 1983).

95% Ethanol	300ml
Chloroform	160ml
90% Lactic acid	125ml
Phenol	150g
Chlorol hydrate	450g
Aniline blue	0.6g

Concentrated chlorol hydrate solution was prepared by dissolving 250g of chlorol hydrate in 100ml of distilled water.

3.2.3.2. Preparation of inoculated tissue for light microscopy.

Sections of the inoculated leaf tissue approximately 1cm² were removed from beneath inoculation droplets using a Disel cork borer number 5 after 4, 8, 12, 48 hours and thereafter on a daily basis until the 15th day. The tissues were cleared and stained using Bruzzese and Hasan clearing and staining technique (Bruzzese and Hasan,1983). The tissues were immersed in the clearing-staining solution (2ml/cm² of tissue) in stoppered glass vials for 48 hours at room temperature (20-24°C). The tissues were then removed and placed for 12-48 hours in the concentrated chloral hydrate solution (2.5 g/ml water) and finally rinsed in distilled water. They were then mounted in clear lactophenol on a microscope slide for viewing under the light microscope.

Observations were made on the mode of conidial germination, penetration and colonization of the host cells by *P. griseola*. The number of synnemata per lesion and the number of conidiophores per synnema were counted. A completely randomized design with 10 replicates was used. The values obtained were transformed using the square root transformation and analysed statistically.

3.2.3.3. Preparation of transverse sections of the inoculated tissues.

Semi-thin sections (1.0-1.5µm) of resin embedded material prepared as for transmission electron microscope (section 3.2.4.) were mounted and stained at 60°C for one minute with 1% (w/v) toluidine blue in 1% borax solution. The sections were viewed

under the light microscope and photographs taken where appropriate.

3.2.4. Transmission electron microscopy

3.2.4.1 Preparation of fixatives and buffers.

Glutaraldehyde at 2.5% (w/v) concentration buffered in 0.05M sodium cacodylate (pH 7.2) was prepared by mixing 5 ml of 25% glutaraldehyde, 2.5 g of sucrose, 5 ml of 0.1M calcium chloride and 12.5 ml of 0.2M sodium cacodylate buffer (pH 7.2) prepared by dissolving 10.7 g of sodium cacodylate in 250 ml of distilled water. 0.05M sodium cacodylate buffer was prepared by mixing 62.5 ml of 0.2M sodium cacodylate with 187.5 ml of distilled water. Osmium tetroxide at 3% (w/v) concentration in 0.05M sodium cacodylate buffer was prepared by mixing 7.5 ml of 4% Osmium, and 2.5 ml of 0.2M sodium cacodylate buffer (pH 7.2). A saturated solution of Uranyl acetate in 70% alcohol was prepared by dissolving Uranyl acetate in 70% alcohol until no more could dissolve.

3.2.4.2 Preparation of dehydrating solutions.

Varying concentrations of ethanol (50%, 70%, 80% and 96%) were prepared by diluting absolute ethanol in distilled water to the desired concentrations.

3.2.4.3 Preparation of embedding media.

Araldite mixture was prepared by mixing 10 ml of Araldite CY212, 10 ml of Dodecanyl succinic anhydride (DDSA), 0.4 ml of Benzyl dimethyamine (BDMA) and 0.3 ml of Dibutylphthalate, taking care not to introduce air bubbles.

3.2.4.4. Preparation of stains.

Alkaline lead citrate at 0.2% concentration was prepared by dissolving 0.8 g lead citrate in 4 ml of distilled water and mixing it with 0.04 ml sodium hydroxide prepared by dissolving 0.25 g of sodium hydroxide pelletes in 2.5 ml of distilled water. The solution was filtered through a sterile 200nm millipore filter into disposable plastic syringe with a needle attached ready for use.

1% Toluidine blue in 1% Borax solution was prepared by dissolving 1 g of toluidine blue in 100 ml of 1% aqueous borax solution.

3.2.4.5. Preparation of inoculated tissues.

Inoculated bean leaf tissues were obtained as in section 3.2.3.2. The tissues were cut into strips of approximately 1mm wide and 3-5mm long in 2.5% (w/v) glutaraldehyde fixative in 0.05M sodium cacodylate buffer (pH 7.2) prepared as in section 3.2.4.1.

3.2.4.6. Fixation

Tissue fixation was done according to the method described by O'connell *et al* (1984). The inoculated tissues were immersed in 2.5% (w/v) glutaraldehyde fixative bufferred in 0.05M Sodium cacodylate (pH 7.2) in stoppered glass vials. The tissues were vacuum infiltrated for 10 minutes and left in the fixative for 4 hours in the fridge, after which they were thoroughly rinsed 3 times in buffer (Sodium cacodylate). They were postfixed in 3%

(w/v) Osmium tetroxide for 30 minutes, thoroughly rinsed in sodium cacodylate buffer and finally fixed for 1 hour in a saturated solution of Uranyl acetate in 70% alcohol.

3.2.4.7. Dehydration

The tissues were passed through a sequence of increasing concentrations of ethanol (50%, 70%, 80%, 96% each for 10 minutes and then through 3 changes of 100% ethanol each for 7 minutes). They were then left in a mixture of Propylene oxide and absolute alcohol in the ratio of 1:1 for 10 minutes and finally in 100% Propylene oxide for 10 minutes.

3.2.4.8. Embedding

The tissues were kept overnight in a 1/2 volume of Propylene oxide and 1/2 volume of Araldite mixture prepared as in section 3.2.4.3. and then transferred to 100% of Araldite mixture. The vials were placed on a Taab rotator for 4 hours to facilitate infiltration of the embedding medium. The tissues were embedded in 100% Araldite mixture in agar block moulds, labelling of tissue was done and the embedding medium was polymerized by heating the moulds in a Memmert oven at 60°c for 2 days.

3.2.4.9. Tissue sectioning and staining.

The blocks with the embedded tissues were trimmed with a Reichert MT.60 block trimmer to about 0.2mm on the longest side of the trapezoid. Semi-thin sections of about 1.0 to 1.5µm were cut using an LKB 111 Ultramicrotome fitted with a glass knife. The sections were mounted on a microscope slide and stained in 1% (w/v) Toluidine blue in 1% Borax solution. The sections were

observed under the Light microscope and blocks with sections showing particular stages of fungal development identified. These blocks were further sectioned and serial ultrathin sections were picked with 400 mesh copper grids and stained with 0.2% alkaline lead citrate for 15 minutes.

3.2.4.10. Viewing and photography of infected tissues

The grids were viewed under the electron microscope and observations made on penetration and colonization of the tissues by *P. griseola*. Where necessary, photographs were taken.

3.2.5. Scanning electron microscopy (SEM).

3.2.5.1. Preparation of infected tissues.

The fixing and dehydrating agents were prepared as in section 3.2.4. Tissues of inoculated bean leaves were obtained as in section 3.2.3.2. and fixed in 2.5% (w/v) glutaraldehyde in 0.05M sodium cacodylate buffer (pH 7.2) for 1 hour at room temperature. They were dehydrated as in section 3.2.4.7., mounted on metal stubs and gold coated.

3.2.5.2. Viewing and photography under the SEM

The specimens were viewed under a Joel JSM-T330A SEM and observations made on conidia germination, penetration and sporulation. Photographs were taken where appropriate.

CHAPTER FOUR

4.0.RESULTS

4.1. Isolation of *P. griseola* isolates

Good sporulation of *P. griseola* on infected bean material was observed 6 days after incubation but isolation was made 4 days after incubation to avoid contamination by saprophytes on the material. The conidia were borne at the tip of conidiophores organized into a bundle form, synnemata. The conidia were brown in colour, cylindrical to spindle shaped, sometimes slightly curved but not constricted (plate 1). They were smooth and septate but varied in size and number of septa. Some Y-shaped conidia were also noted.

4.2. Single spore cultures of *P. griseola* isolates

4.2.1. Slide cultures of *P. griseola*

In each given isolate, conidia varied both in size and shape. The conidia were attached on the conidiophores either as a cluster at the tip or spread out along the conidiophores singly or in twos. Hyphal fusion was common in all the isolates.

4.2.2. Conidial size

Routinely, all the 18 isolates were purified using single spore culture technique. However due to variabality in conidial size observed within and among isolates, 10 isolates were used to determine whether this variability was race-specific. There was considerable variation in conidial length, width and number of septa within the isolates tested. The range of these parameters

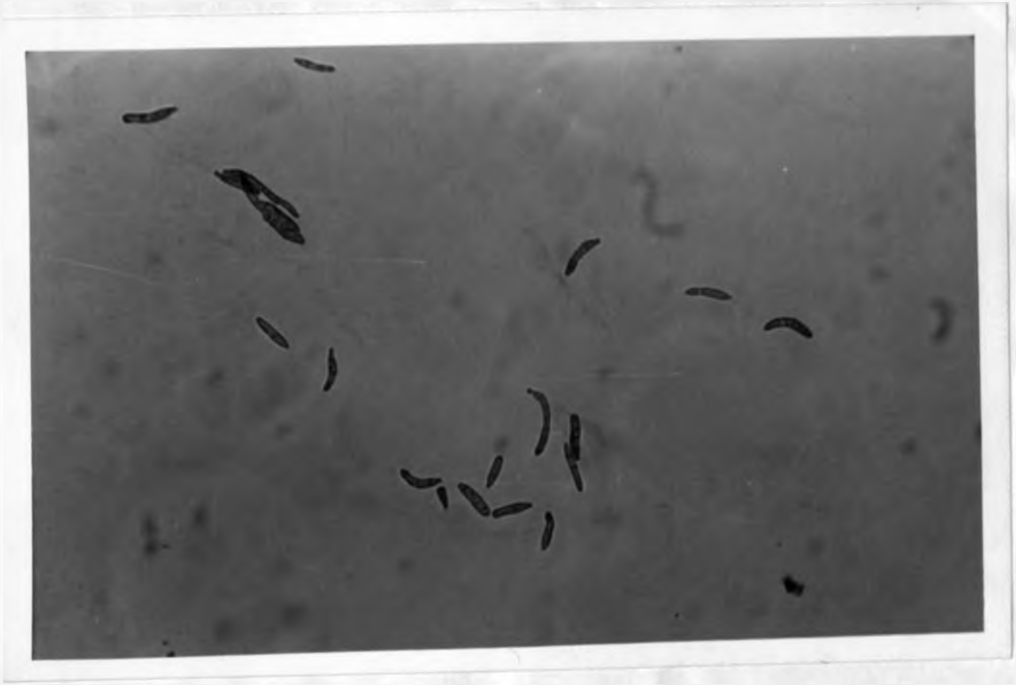

Plate 1: Conidia of *Phaeoisariopsis griseola*

(Magnification X200)

within each isolate was found to be wide (Table 2). The mean conidial length for the ten isolates used was 34.3µ but varied from 19.95 to 71.5µ. However, there was no significant (P=0.05) difference in conidial length among the isolates (appendix 1). The width varied from 2.85 to 14.3µ with an average of 6.74µ. There was a significant difference in conidial width among the isolates (appendix 2). For instance, isolate PG11 had significantly (P=0.05) wider conidia when compared to all the other isolates except isolate PG4. The number of septa varied from 0 to 11 but there was no significant (P=0.05) difference among the isolates with respect to number of septa (appendix 3). 76% of all the conidia measured had 1-3 septa and only 24% of the conidia had more than 4 septa (Table 3). 10% of the conidia had no septa and only 0.53% conidia had 11 septa. The most frequent conidia were those with 3 septa (Table 3). Isolate PG15 had the smallest septation range (0-2) whereas PG11 had the widest range in the number of septa which was 0-11.

Table 2: The mean* and range in conidial length and width in micrometers and number of septa of 10 isolates of *Phaeoisariopsis griseola*

Isolate	Length (µ)		Width (µ)		No. of septa	
	Mean	Range	Mean	Range	Mean	Range
1 PG11	35.6a	22.8-48.5	8.95a	4.3-14.3	3a	0-11
2 PG4	32.8a	22.8-45.6	7.88ab	5.7-11.4	2a	1-4
3 PG1	33.6a	22.8-48.5	6.85bc	2.9-8.6	3a	0-4
4 PG15	32a	19.9-71.3	6.68bc	2.9-9.98	2a	0-2
5 PG2	35.9a	28.5-51.3	6.55bc	2.9-8.6	2a	0-3
6 PG10	37.1a	22.8-51.3	6.55bc	4.3-8.6	3a	0-4
7 PG12	36.2a	22.8-71.3	6.23bc	2.9-11.4	3a	1-5
8 PG7	30.2a	22.8-39.9	6.08bc	4.3-8.55	3a	1-4
9 PG5	34.9a	22.8-51.3	5.88c	4.3-8.55	2a	0-5
10 PG8	34.8a	19.95-57	5.33c	2.85-5.55	3a	0-4

* = Average of 100 conidia

Means followed by the same letter within each column are not significantly different at P=0.05 according to Duncan's New Multiple range test.

Table 3: *Phaeoisariopsis griseola* conidia septa frequency

No. of septa per conidia	0	1	2	3	4	5	6	7	8	9	10	11
Frequency	21	47	43	61	17	10	0	0	0	0	0	1

4.2.3. Growth of *P. griseola* on artificial medium.

Single spore cultures of all the 18 isolates were grown on BLDA. Observations showed some variation in mycelial growth and colouration. Thus, 10 isolates were used to determine if the variations observed were associated with pathogenicity of a given isolate. Growth of *P. griseola* isolates on BLDA was compared on the basis of visual observation of the colour and type of mycelial growth. In all the 10 isolates, growth was greyish brown and fluffy for the first 5 days but later the colour changed to grey and in some isolates, it finally changed to black. Isolates PG5, PG7, PG8, and PG10 produced grey mycelia in contrast to the blackish mycelia found to be characteristic of isolates PG2, PG4, PG1, PG11, PG12 and PG15.

Small white, fluffy or grey sectors appeared in cultures of most isolates and they increased with increased period of incubation at 24°c. However, this sectoring was more prominent in some isolates than in others. For example, isolates PG15, PG11 and PG1 exhibited alot of sectoring whereas no sectoring was observed at all in isolates PG4, PG8 and PG2. Some of the cultures had condensed moisture below the lids of the petri-dishes, for example isolates PG7, PG15, PG11 and PG5.

Isolates PG1, PG10, PG11 and PG12 exhibited fluffy growth but isolates PG2, PG4, PG5, PG7, PG8 and PG15 had smooth compact mycelial growth. However, after subculturing for about 5 times, some isolates changed their growth habit. For example isolates PG4 and PG15 produced fluffy grey mycelia with a lot of sectoring. These cultures which were initially blackish, also had fewer conidia as compared to those with blackish coloured mycelia and took more than 10 days to sporulate.

4.3. Pathogenicity test

The first symptoms were observed on the primary leaves 4 days after inoculation. The lesions were initially circular, light coloured but later turned greyish to bluish grey (Plate 2). On the trifoliolate leaves, small angular lesions delimited by veins and veinlets appeared and were found to vary in size (Plate 3). In some cases, the lesions coalesced to produce aggregate lesions of greater dimensions. In both the primary and trifoliolate leaves, the symptoms appeared first on the lower leaf surface as light grey lesions.

As the disease progressed, dark specks of columnar structures (synnemata) were visible on the lesions on the underside of the leaf with the naked eyes. When the synnema was observed under the microscope, the conidiophores were seen to be closely packed together, straight at the bottom but divergent at the tips. The conidia borne at the tip of conidiophores were pale brown, cylindrical to spindle shaped, smooth, septate and sometimes curved.

Plate 2: Angular leafspot symptoms appearing on an old defoliated primary leaf of cultivar M29 as bluish grey circular lesions.

Plate 3. Angular leafspot symptoms on primary (P) and trifoliolate (T) leaves of cv M29

Brown angular lesions were also apparent on the upper surfaces of the leaf but the synnemata were effectively confined to the lower surface. In the advanced stages of the disease, leafspots coalesced causing extensive necrosis, chlorosis, leaf curling, drying and eventually defoliation. All isolates except PG1 induced typical ALS symptoms on cvs Rosecoco-GLP-2 and M29. Isolate PG1 induced susceptible ALS symptoms on cv M29 and a resistant type of reaction on cv Rosecoco-GLP-2, thus cv M29 seemed to serve as a universal suscept in these studies. The resistant reaction on cv Rosecoco-GLP-2 was exhibited as chlorosis on the inoculated leaves 6 days after inoculation and the chlorotic leaves eventually defoliated after 2 weeks.

4.4. Pathogenic variation in *P. griseola*

4.4.1. Angular leafspot symptoms on bean leaves.

4.4.1.1. ALS symptoms in the compatible interactions of *P. griseola* and bean cultivars.

The symptoms induced by *P. griseola* isolates varied in terms of size and time of appearance depending on the isolate and cv used. For instance, in the compatible interaction of cv Rosecoco-GLP-2 and isolate PG2, ALS symptoms first developed on primary leaves 4 days after inoculation. The lesions were initially circular and light coloured but later turned grey to bluish grey. These lesions enlarged very fast and by the 8th day, they had attained larger sizes. The leaf parts which were not infected became chlorotic and later dried up attaining a brownish paperly appearance.

The symptoms on the trifoliolates ranged from small lesions clearly delimited by veins and veinlets to large coalescing

lesions of greater dimensions. For example, cv Rosecoco-GLP-2 developed large lesions which coalesced starting from the 8th day after separate inoculations with isolates PG2, PG12 and PG15. However, the characteristic lesions which developed on cv G1805 when inoculated with isolate PG1 did not coalesce even as the disease progressed. On the other hand, some lesions coalesced even though the percentage leaf area covered by lesions was low like in the case of cv M16 when inoculated with isolate PG7. Isolate PG1 induced small clearly delimited lesions on all the susceptible cultivars whereas isolate PG16 caused large coalescing lesions surrounded by a spreading burning reaction which later became chlorotic on all the cultivars susceptible to it as exemplified by cultivars A339 and Rosecoco-GLP-2.

Heavy sporulation was observed on the lower surface of the leaves 10 days after inoculation and heavily infected leaves defoliated by the 19th day. Chlorosis followed by premature defoliation occurred in some of the compatible interactions like in the case of cultivars E2 and Mwitemania-GLP-x.92 when inoculated with isolate PG16 and this occurred 3 weeks after inoculation.

4.4.1.2. ALS symptoms in the moderate resistance interactions.

The ALS symptoms on the moderately resistant cultivars were similar to those induced on the highly susceptible cultivars and only differed in size and time of appearance. For example when cv M29 was inoculated with isolate PG3, lesions developed on the 8th day and were smaller (covering only 5.67%) than those on the highly susceptible cvs like Mwitemania-GLP-x.92 which covered as

much as 30% of the leaf area. These lesions coalesced on the 10th day after inoculation and sporulation occurred 2 days later. Leaf defoliation in this cv occurred 24 days after inoculation.

4.4.1.3. The incompatible interactions of *P. griseola* and bean cultivars.

In the incompatible interactions, the reactions varied depending on the isolate and cultivar. Some interactions produced tiny necrotic flecks whereas in others, chlorosis and tiny non-sporulating lesions developed. For example, when cvs G9603 and A285 were inoculated with isolates PG1 and PG5, only chlorosis was observed. Isolates PG4, PG5 and PG11 induced tiny necrotic flecks on cv M26 whereas when the same cv was inoculated with isolates PG7 and PG10, only chlorosis occurred. The same cultivar developed tiny non-sporulating lesions covering about 2.5% of the leaf area when inoculated with isolate PG17. The incompatible interaction of cv Montcalm and isolate PG13 was characterised by non-sporulating lesions covering about 4.7% of the leaf area 15 days after inoculation.

4.4.2. Race typing of *P. griseola*

Angular leafspot symptoms development on both the primary simple and first trifoliolate leaves was observed on a daily basis up to 3 weeks. However ALS severity assessment was made 14 days after inoculation (except for plants inoculated with isolates PG1 and PG6 which was after 21 days) and was based on a CIAT evaluation scale of 1 to 9 representing varying percentages of actual leaflet area covered by lesions (Plate 4). The reactions of the 30 bean cvs/lines inoculated with different

Plate 4. Infected bean leaves showing grades 3, 5, 7 and 9 of the disease severity scale used in ALS disease assessment.

(a) Cultivar Rosecoco-GLP-2 leaves showing ALS disease grade 3 after inoculation with isolate PG1.

(b) Cultivar Caraota leaves showing ALS disease grade 5 after inoculation with isolate PG1.

(c) Cultivar M26 leaves showing ALS disease grade 7 after inoculation with isolate PG1.

(d) Cultivar M14 leaves showing ALS disease grade 9 after inoculation with isolate PG15.

a

b

c

d

isolates varied considerably ranging from immune (disease severity index 1) to very susceptible (disease severity index 9).

All the 18 isolates were pathogenic and interacted differentially with the 30 bean cvs/lines (Table 4 and 5). Some of the isolates induced very susceptible reactions on some bean cvs whereas on others the same isolates were not infective at all. For instance, isolates PG2, PG7, PG8 and PG15 induced a susceptible reaction on bean cvs Calima (38%, 42%, 15% and 28% disease respectively) and Montcalm (25%, 27%, 10% and 21% disease respectively). However, only PG2 and not any of the other 3 isolates induced a susceptible reaction on cvs Caraota and BAT76 (Table 4). On cv Caraota, isolates PG2, PG7, PG8 and PG15 induced 8%, 0%, 0.3% and 2% disease respectively whereas on cv BAT76 they caused 11%, 0%, 0.3% and 1.3% disease respectively. Cultivars A235 and G2858 were resistant to all the isolates except to PG1 and PG8. Isolate PG1 induced 7% and 6.7% disease respectively on the two cvs whereas PG8 induced 9% and 14% respectively. None of the 22 CIAT differentials was susceptible to all the isolates. Most of them were resistant unlike the local cvs which were susceptible to most of the isolates. Line M29 was susceptible to all the 18 isolates whereas line M26 was resistant to 15 of the isolates used. The popularly grown bean cvs in Kenya, cvs Rosecoco-GLP-2 and Mwitemania-GLP-X.92 were susceptible to all the isolates except to PG1, and PG1 and PG17 respectively. CIAT differentials G9603 and A285 were resistant to all the 18 isolates.

Table 4 : Differential reactions of *Phaseolus vulgaris* cultivars and lines inoculated with 18 isolates of *Phaeoisoriopsis griseola* Sacc.

Bean cultivars.	*P. griseola* isolates																	
	PG1	PG2	PG3	PG4	PG5	PG6	PG7	PG8	PG9	PG10	PG11	PG12	PG13	PG14	PG15	PG16	PG17	PG18
1. BAT 76	S	S	S	R	R	R	R	R	R	S	R	R	S	R	R	R	R	R
2. Calima	S	S	S	R	S	R	S	S	S	S	R	S	S	R	S	R	S	S
3. Montcalm	R	S	R	R	S	S	S	S	S	S	S	S	R	S	S	R	S	S
4. A62	R	R	S	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R
5. BAT 332	S	R	S	S	R	R	R	R	R	R	R	S	S	R	R	S	R	R
6. G5686	R	S	R	R	R	S	R	R	R	S	R	R	R	R	S	R	R	R
7. CARAOTA	S	S	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	S
8. SEAFARER	S	R	R	R	R	R	R	R	S	R	R	S	R	R	S	R	R	R
9. ALABAMA NO. 1	S	S	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R	R
10. G9603	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
11. BAT 1647	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
12. A21	S	S	S	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R
13. P. CHECHA	S	S	S	R	R	R	R	S	R	R	R	R	S	R	S	R	R	R
14. A 235	S	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
15. G1805	S	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	S
16. A212	S	R	R	R	R	R	R	R	R	S	R	S	R	R	S	R	R	R
17. AMENDOIN	R	S	S	S	R	S	R	S	S	S	S	R	S	S	S	S	S	R
18. G2858	S	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
19. A301	S	R	S	S	R	R	R	S	S	R	R	S	S	R	R	S	R	R
20. A339	S	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R	S	R
21. C49242	R	S	S	R	R	R	R	S	R	R	R	R	S	R	S	R	R	S
22. A285	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
23. Rosecoco-GLP-2	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
24. M16	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S
25. GLP-X.92	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
26. M26	S	S	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
27. M29	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
28. E2	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
29. M14	S	S	S	R	S	R	S	S	S	R	S	S	S	S	S	R	S	S
30. M20	R	S	R	R	R	R	R	R	S	R	R	S	R	R	R	R	R	S

Table 5: THE ANGULAR LEAFSPOT SEVERITY MEANS ON 30 BEAN CULTIVARS SEPARATELY INOCULATED USING EIGHTEEN *P. GRISEOLA* ISOLATES.

CULTIVARS	ISOLATES																		
	PG1	PG2	PG3	PG4	PG5	PG6	PG7	PG8	PG9	PG10	PG11	PG12	PG13	PG14	PG15	PG16	PG17	PG18	Variety means
1. BAT 76	5.67	11.0	12.33	0.00	1.00	0.67	0.00	0.33	0.33	5.67	1.67	2.00	11.67	0.67	1.33	0.67	0.67	0.00	3.09i-m
2. Calima	15.00	38.33	8.00	1.33	21.00	0.67	41.67	15.00	15.00	34.33	1.67	2.67	6.67	1.67	28.33	2.00	31.67	23.33	16.02cd
3. Montcalm	2.00	25.00	0.67	1.33	11.00	5.67	26.67	10.00	11.67	42.33	10.00	4.67	4.67	5.00	20.67	0.00	37.67	6.67	12.31e
4. A62	1.67	0.00	15.67	0.00	0.00	2.33	0.67	0.00	0.00	0.00	0.00	0.00	11.00	1.00	0.00	1.33	7.00	1.00	2.3mn
5. BAT 332	11.67	1.00	12.67	16.67	1.00	0.00	0.33	2.00	2.33	0.00	0.67	22.67	2.33	1.33	0.67	8.33	1.67	1.67	4.83gh
6. G5686	1.00	23.33	0.33	1.00	0.00	6.33	0.33	2.00	0.33	10.00	0.00	1.33	0.00	1.33	16.67	2.67	1.00	1.00	3.67h-l
7. Caraota	5.33	8.33	1.00	0.00	2.67	0.33	0.00	0.33	1.33	6.67	1.67	0.67	1.00	1.67	1.67	0.33	0.00	10.00	2.39j-m
8. Seafare	7.33	1.33	1.67	0.33	2.00	0.00	0.00	2.00	16.67	0.00	0.00	2.33	0.67	1.33	14.00	1.67	2.00	1.67	3.06i-m
9. Alabama No. 1	19.33	13.33	0.00	0.33	1.33	2.00	0.33	6.33	0.00	0.33	3.33	0.33	1.67	0.67	8.67	1.00	0.00	0.67	3.26j-m
10. G9603	0.00	2.33	1.00	0.00	0.00	0.33	0.66	0.67	1.33	1.00	0.67	1.67	1.67	1.67	0.67	1.33	0.67	1.67	0.96n
11.BAT 1647	19.00	15.00	1.33	0.00	0.67	1.00	0.00	0.33	2.33	0.00	2.33	0.67	1.67	1.67	1.67	0.67	1.33	0.00	2.76j-m
12. A21	5.00	7.67	20.00	0.67	2.00	1.67	0.33	0.67	0.33	2.00	2.33	1.33	11.00	0.00	1.67	1.00	1.33	1.67	3.37g-j
13. P. checha	5.33	18.33	16.00	0.00	0.33	0.00	1.67	16.33	2.00	0.00	0.33	0.00	8.33	2.00	10.67	1.00	1.67	2.33	4.80jhi
14. A 235	7.00	2.33	0.33	2.00	2.00	0.33	0.00	9.00	0.00	1.33	1.67	0.00	1.33	0.67	2.00	0.33	1.33	1.67	1.85lmn
15. G1805	20.67	1.33	0.33	1.00	1.67	0.33	2.67	0.00	0.00	7.33	0.00	1.67	1.33	1.00	1.33	2.00	0.00	5.67	2.69j-m
16. A212	5.33	0.00	0.67	0.00	1.33	0.67	1.67	1.00	1.67	16.67	4.00	10.00	1.33	1.33	9.00	1.67	2.00	0.33	3.26h-k
17. Amendoin	1.00	45.67	11.67	13.33	0.67	7.33	0.00	6.33	16.67	11.00	6.67	0.00	9.00	8.00	6.67	14.00	21.67	2.33	10.11f
18. G2858	6.67	1.33	0.67	0.33	0.33	1.67	0.33	14.00	0.00	0.33	1.33	2.33	0.67	0.67	2.00	1.67	0.00	2.00	2.02k-n
19. A 301	19.67	0.67	12.67	11.67	0.00	0.00	0.33	9.33	7.33	1.00	1.00	4.00	13.33	1.33	0.00	12.00	0.33	1.00	5.26g
20. A 339	8.67	2.00	0.67	10.67	2.00	0.69	0.00	0.00	19.00	1.67	0.67	0.00	2.33	1.67	1.00	17.33	1.67	0.67	3.93g-j
21. C49242	0.33	5.00	7.67	0.00	2.00	1.67	1.00	15.00	0.00	2.00	2.00	2.33	16.67	0.33	5.00	1.00	0.00	11.67	4.65gh
22. A285	0.00	0.00	0.00	0.67	0.00	0.00	0.00	0.33	0.33	0.00	0.00	0.33	1.67	0.00	0.00	0.33	0.00	2.33	0.330
23. GLP-2	1.67	53.67	41.00	17.33	28.33	9.00	36.67	40.00	35.00	23.67	17.33	19.33	36.00	40.00	8.33	38.33	26.67	26.00	27.69a
24. M16	7.00	9.00	26.67	1.67	10.33	14.00	7.33	18.67	21.67	10.00	9.67	12.33	18.33	9.00	0.33	1.67	8.00	8.00	10.76e
25. GLP-X.92	1.33	31.67	31.67	31.67	9.00	16.67	10.00	28.33	19.00	8.00	10.67	23.33	26.67	11.33	7.67	47.33	2.33	21.00	18.76b
26. M26	14.67	26.67	0.33	1.00	0.00	0.67	0.67	0.33	0.33	0.00	0.33	2.00	0.67	1.33	12.33	0.67	2.33	1.67	3.67j-m
27. M29	6.33	51.00	5.67	10.67	20.00	8.33	18.33	7.33	17.33	16.67	20.67	10.67	7.67	18.33	9.00	41.67	35.00	8.00	17.37b
28. E2	0.67	26.67	8.33	24.00	19.67	0.00	10.67	12.67	16.67	41.67	6.67	18.33	6.67	7.33	20.00	9.00	10.67	19.33	14.39c
29. M14	5.00	10.33	21.00	0.00	13.67	1.67	18.33	11.69	21.67	1.33	27.67	15.67	19.33	18.33	21.67	0.33	11.00	13.33	12.89de
30. M20	2.33	11.67	0.00	1.33	0.00	0.67	0.00	1.00	9.67	2.00	0.00	9.00	1.33	0.33	1.33	1.33	1.67	10.67	3.02i-m
Isolate means	6.89 h	15.13 a	8.67 b	4.97 g	5.13 g	2.82 h	6.02 g	7.70 bc	8.00 bc	8.23 bcd	4.43 g	5.72 ef	7.42 bc	4.70 fg	7.14 bc	7.08 de	7.07 e	6.21 cde	

Means followed by the same letters within a row or column are not significantly (P= 0.05) different according to Duncan's New Multiple range test.

Isolate PG1 and PG6 were less aggressive than the others with isolate PG6 being the least aggressive. Isolate PG1 caused the first symptoms on susceptible cvs 15 days after inoculation whereas PG6 only produced very tiny lesions on the susceptible cvs 19 days after inoculation. These two isolates induced the least amount of angular leafspot disease on the leaves even on the most susceptible cvs. For instance PG6 induced 17% and 9% disease on cvs Mwitemania-GLP-X.92 and Rosecoco-GLP-2 respectively. Isolate PG1 did not induce a susceptible reaction on either cv Mwitemania-GLP-X-92 or Rosecoco-GLP-2. However PG1 induced a susceptible reaction on cv G1805 with an average angular leafspot of 20.7% which was the highest amount of disease induced by this isolate. Isolate PG6 induced susceptible reactions on 7 cultivars whereas only 11 cultivars were resistant to isolate PG1. Isolates PG2, PG7, PG10 and PG17 were the most aggressive and induced the first symptoms 6 days after inoculation.

The 18 isolates used were grouped into 15 races when the data on the type of reactions elicited was used (Table 4). However, when the actual ALS severity data was subjected to statistical analysis and Duncan's New multiple Range Test used to separate the isolate means (Table 5), the scenario changed slightly in that the 18 isolates were grouped into 11 races but the former grouping was adopted (Table 6). The isolates, cultivars and their interactions were significantly different at 0.1% level (appendix 6).

Apart from 6 isolates which could be grouped into 3 races, each of the other isolates was grouped into its own race according to the reaction they elicited on the 30 differentials.

Table 6: Physiological races of *P. griseola*

Isolate	Area of origin	Race
PG1	Kangema	1
PG2	Ndeiya	2
PG3	Thika (K.A.R.I.)	3
PG4	Wambugu Farm	4
PG5	Mweiga	5
PG6	Kerugoya	6
PG7	Ndaragwa	5
PG8	Josa	7
PG9	Weruga	8
PG10	Embu (K.A.R.I.)	9
PG11	Katumani (K.A.R.I.)	10
PG12	Chuka	11
PG13	Bahati	12
PG14	Ngong	10
PG15	Keumbo (Kisii)	13
PG16	Sabatia	4
PG17	Embakasi	14
PG18	Kabete	15

Isolate PG4 from Wambugu farm in Nyeri district and isolate PG16 from Sabatia in Vihiga district were grouped together since they induced similar reactions on the cultivars. Isolate PG5 from Mweiga in Nyeri district and PG7 from Ndaragwa in Nyandarua district were both placed into race 5. However isolate PG5 caused a hypersensitive reaction exhibited as tiny necrotic flecks on cvs M26 and M20 whereas isolate PG7 only induced flecks on cv A235.

Even isolates from similar geographical regions varied in their pathogenicity when inoculated on the 30 cvs/lines. For example, the two isolates from Nyeri district were pathogenically different and only induced similar reactions on some but not all the cultivars. Isolates from the cold areas, for example upper parts of Murang'a, Kerugoya and Nyeri districts also showed variation in their pathogenicity towards the differentials. For instance, isolate PG1 infected 19 cvs whereas PG4, PG5 and PG6 infected 8, 8 and 7 cvs respectively. Isolates PG4, PG5 and PG6 induced a susceptible reaction on cv Rosecoco-GLP-2 (17.3%, 28.3% and 9% disease respectively) but PG1 induced a resistant reaction (1.7% disease) on the same cv. Isolate PG1 induced small delimited lesions on all the susceptible cvs whereas the other isolates induced either delimited or coalescing lesions depending on the cv. However, isolates from Katumani and Ngong which are relatively hot areas induced similar reactions on all the cultivars but there existed some variation with respect to the amount of disease and type of lesions they induced on the susceptible cultivars. For example, isolate PG11 from Katumani in Machakos district induced a very susceptible reaction on cultivar M14 (28%) whereas isolate PG14 from Ngong in Kajiado district

induced a susceptible reaction with 18% disease on the same cultivar.

4.5. Bean tissue colonization by *Phaeoisariopsis griseola* Sacc.

4.5.1. Light microscopy.

4.5.1.1. Pre-penetration and penetration events of *P. griseola*.

Observations made on the inoculated, cleared and stained bean leaf tissues showed that spore germination on cvs Rosecoco-GLP-2 (susceptible) and M29 (intermediately resistant) occurred 4 hours after inoculation using isolate PG18 and germtubes were produced at either one or both tips of the conidia while others emerged from the sides. Prior to germination, the spores increased in length and more so in width. They stained heavily with lactophenol in cotton blue and the septa were more conspicuous than on the ungerminating spores. The germtubes had no appressoria but produced tiny penetration pegs at their tips 12 hours after germination. On cv M26, most of the conidia had not germinated 4 hours after inoculation and their sizes had not changed. The germinated conidia on this cv had shorter germtubes than those on cvs Rosecoco-GLP-2 and M29. By 24 hours after inoculation, no ungerminated conidia were observed on cvs Rosecoco-GLP-2 and M29 but most of the conidia on cv M26 had not germinated.

Penetration of *P. griseola* occurred 24 hours after inoculation in all the three cultivars. Infection pegs penetrated into the host cells either directly or through the stomata. Some germtubes seemed to be by-passing the stomata and then penetrating directly into the host cell.

4.5.1.2. Colonization of the bean tissue by *P. griseola*

4.5.1.2.1. Colonization of cvs Rosecoco-GLP-2 and M29.

Three days after inoculation, one or more primary hyphae could be seen growing out of infection vesicles in the penetrated cells and progressed both intercellularly and intracellularly. The hyphae branched 4 hours later giving rise to small diameter secondary hyphae which often branched profusely and extensively colonized the surrounding cells. Conspicously septed primary and secondary hyphae were observed on the inoculated area four days after inoculation (Plate 5). The septation was more closely spaced and highly conspicous on cv Rosecoco-GLP-2 than on M29. The diameter and septation of the hyphae decreased with increase in distance from their origin. A prolonged period of biotrophic development was noted and no necrosis occurred until after the 5th day. In these two cultivars although infected cells and the adjacent ones eventually died, they did not immediately become brown and lesions appeared after 6 and 10 days respectively, associated with growth of secondary mycelia from the primary hyphae. After the onset of browning on the 6th day, the growth of the fungus increased rapidly as more secondary hyphae branched out from the primary hyphae and radiated into the surrounding tissue. Browning of the cells occurred in advance of fungal colonization.

Six days after inoculation, development of a more extensive mycelia with highly conspicous septation was observed in cvs Rosecoco-GLP-2 and M29. On cv Rosecoco-GLP-2, the inoculated area became pale brown and individual mycelia had merged to form a spreading lesion. The large diameter primary hyphae occupied the centre of the lesion whereas the smaller diameter secondary

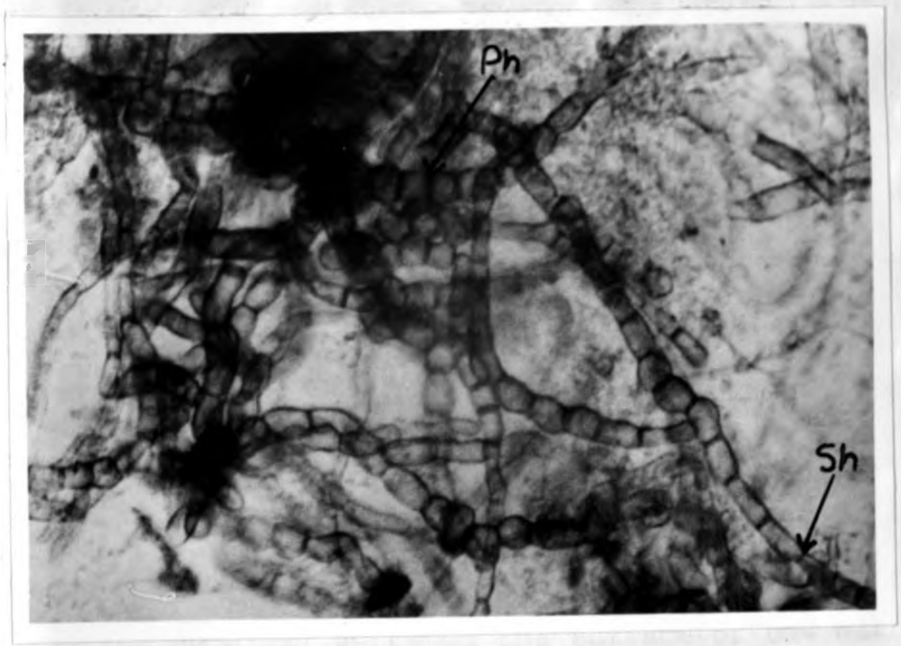

Plate 5. Light micrograph of cleared leaf tissue of cv Rosecoco-GLP-2 showing closely and conspicously septed large diameter primary hyphae (Ph) near the centre and small diameter secondary hyphae (Sh) at the margin of the lesion 4 days after inoculation (Magnification X500).

hyphae spread towards the margin. The highly marked and close septation in the tissue was not observed in culture where septa were far apart and less conspicous. A transverse section of the infected tissue on the 9th day showed cell disintegration. Colonization was both intercellular and intracellular (Plate 6) and was confined more to the lower epidermal and spongy mesophyll region where it was only restricted by vascular bundles whereas the cells towards the palisade region were intact. Complete disruption of the membranes and cytoplasm of the colonized cells was evident on the 15th day (Plate 7). In the colonized cells, hyphae grew within host cells or along the middle lamella and progressed to the surrounding tissue. The margin of the lesion was slightly in advance of the hyphal tips and uninfected cells at the margin of the spreading lesions in the two cvs had disintegrated. After extensive colonization of the tissues, ramifying hyphae emerged from the tissues through tears in the cuticle and formed mycelial wefts on the surface of the mature lesions.

4.5.1.2.2. Colonization of cv M26 by *P. griseola*

On cv M26, infection vesicles were observed 4 days after inoculation with isolate PG18 and short primary hyphae could be seen growing out of them. The hyphae progressed more slowly than on cvs Rosecoco-GLP-2 and M29 and was not as conspicously septed as in the 2 cultivars (Plate 8). The biotrophic development of isolate PG18 in this cv was short and on the 4th day, browning and granulation of the penetrated epidermal and a few of the adjacent uninfected cells was noted. The intensity of browning increased in the next 24 hours and on the 5th day after

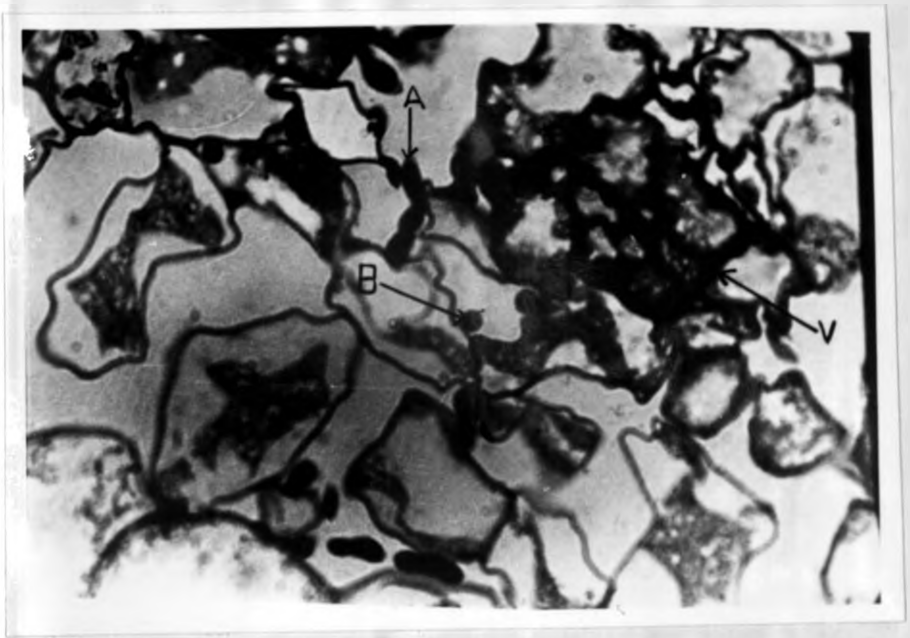

Plate 6. Transverse section of a spreading lesion in cv Rosecoco-GLP-2 showing colonization by intercellular (A) and intracellular (B) hyphae (Magnification X1,250).

V - vascular bundle.

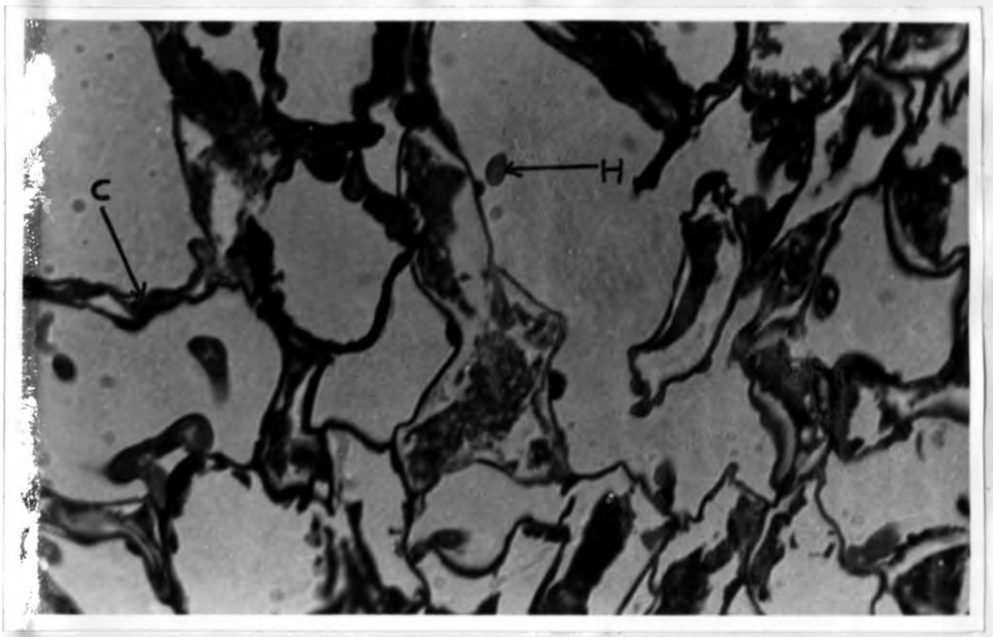

Plate 7. Transverse section of a spreading lesion in cv Rosecoco-GLP-2 showing complete disruption of cells 15 days after inoculation.
(Magnification X1,250).
H - Hyphae
C - disintegrated cells.

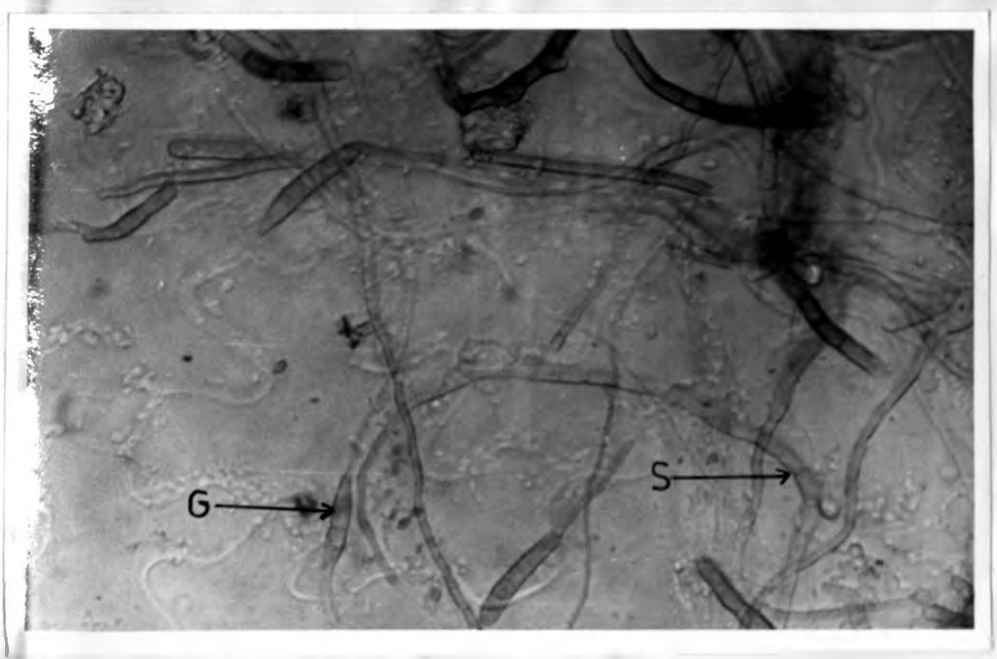

Plate 8. Cleared leaf tissue of cv M26 under the light microscope showing slightly less septed hyphae (S) four days after inoculation with isolate PG18. (Magnification X500).

G - germinating conidia

inoculation, minute brown flecks (small groups of dead cells) were visible on the chlorotic inoculated area.

On the 6th day, the highly septed hyphae observed on cvs Rosecoco-GLP-2 and M29 was also noted on cv M26 although it was not as extensive and only colonized the penetrated and a few of the adjacent cells. The inoculated area appeared chlorotic but a transverse section made on the 9th day showed that the cells were still intact with no evidence of damage to the host cell wall (Plate 9). Only a few fungal structures were present within and between host cells and were confined to the lower epidermal and spongy mesophyll cells. By the 15th day, some of the conidia had still not germinated and the inoculated area had less highly septed hyphae.

4.5.1.3. Sporulation on cultivars Rosecoco-GLP-2 and M29

On the 8th day, stromata had started forming in the lesions on the under surface of the leaf in cv Rosecoco-GLP-2 and were fully formed by the 10th day after inoculation. Dark clusters of conidiophores (synnemata) were visible on these lesions. On cv M29, smaller lesions than on cv Rosecoco-GLP-2 were visible on the 10th day but did not sporulate until the 12th day. The number of synnemata per lesion and that of conidiophores per synnema was significantly (P=0.01) more in cv Rosecoco-GLP-2 than in cv M29 (appendix 4 and 5). In cv Rosecoco-GLP-2, the number of synnemata per lesion and number of conidiophores per synnema ranged from 30-52 and 48-106 respectively whereas on cv M29, they ranged from 5-20 and 15-43 respectively (Table 7).

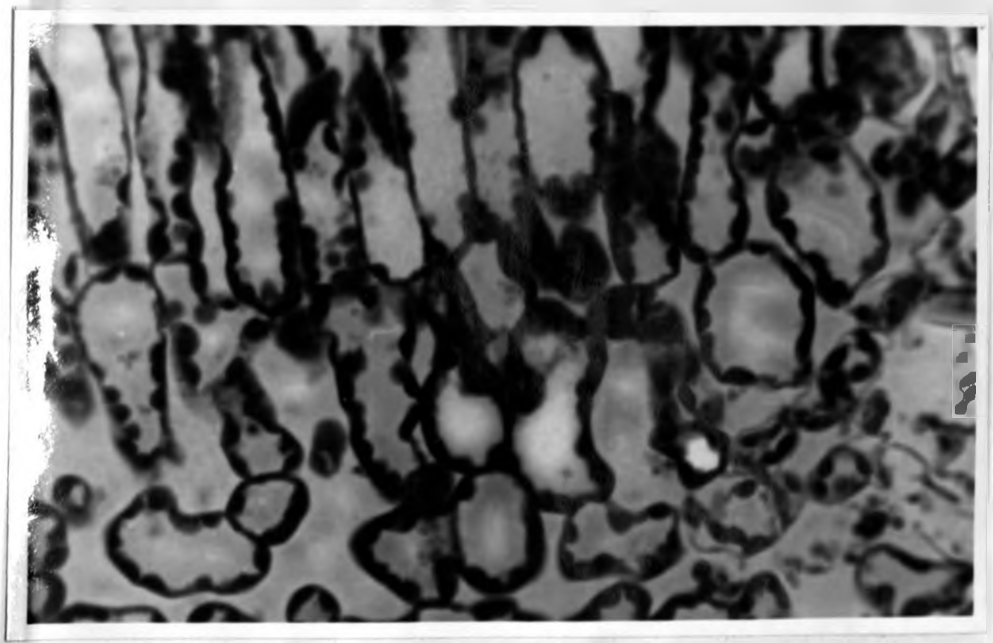

Plate 9. Transverse section of inoculated area on cv M26 showing intact cells 8 days after inoculation with isolate PG18 (under the light microscope at Magnification X500).

Table 7: Number of synnemata per lesion and number of conidiophores per synnema on cultivars Rosecoco-GLP-2, M29 and M26 fifteen days after inoculation with *P. griseola* isolate PG18.

Cultivar	No. of synnemata/lesion		No. of conidiophores/synnema	
	mean	range	mean	range
Rosecoco-GLP-2	41	30-52	72	48-106
M29	13	5-20	30	15-43
M26	0	0	0	0
CV	25.9%		36.2%	
SE	3.7		5.9	
	1%		5%	
$LSD_{(synnemata)}$	5.77		4.28	
$LSD_{(conidiophores)}$	15.5		11.49	

4.5.2. Transmission electron microscopy.

4.5.2.1. Penetration events on the three cultivars.

A transverse section of the host tissues inoculated with isolate PG18 showed that infection pegs penetrated into the host cells either directly or through the stomata (Plate 10). In the case of direct penetration, depression of the cuticle was observed at the point of penetration but no other cell injury was observed (Plate 11).

4.5.3. Scanning electron microscopy.

4.5.3.1. Tissue colonization and sporulation on the three cultivars.

Observations made on the host tissues inoculated with *P. griseola* isolate PG18 showed that tissue colonization was more extensive on cvs Rosecoco-GLP-2 and M29 than on cv M26. On cvs Rosecoco-GLP-2 and M29, synnemata started forming on the 8th and 12th day respectively and were fully formed by the 10th and 15th day respectively. The number of synnemata per lesion was more on cv Rosecoco-GLP-2 than on cv M29 and the synnemata that developed on cv Rosecoco-GLP-2 had more conidiophores than those on cv M29 (Plate 12). In cv M26, no synnemata were observed and the inoculated area had many ungerminated conidia.

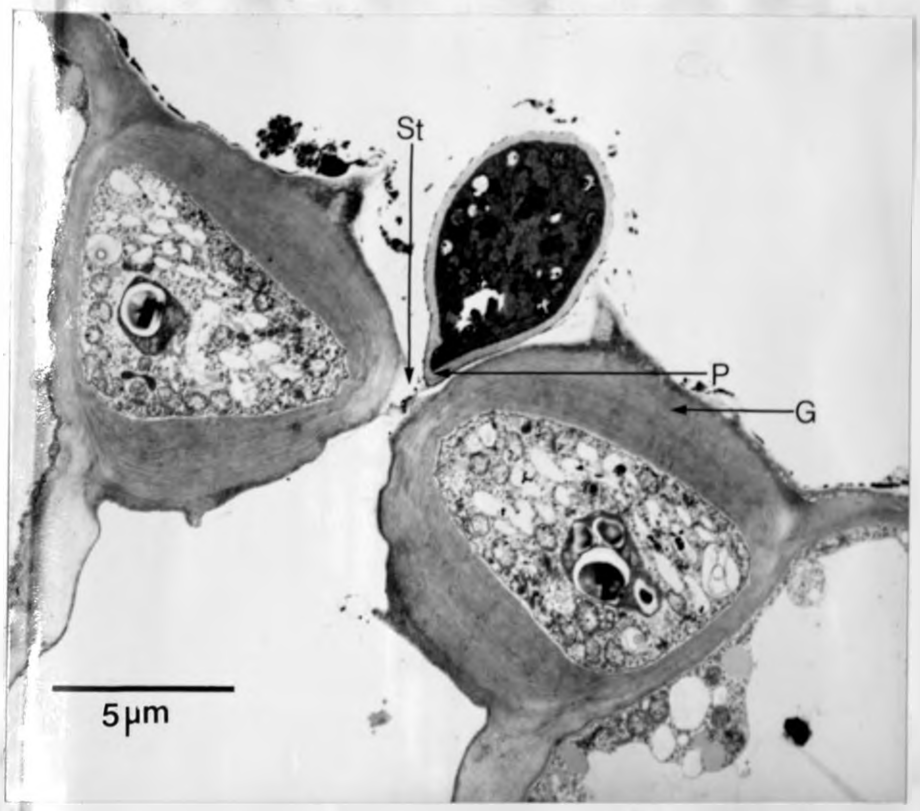

Plate 10. Transmission electron micrograph showing stomatal penetration of bean tissues by *P. griseola*.
(Magnification X49,500)
G - guard cell
P - penetration peg
S - stomatal opening

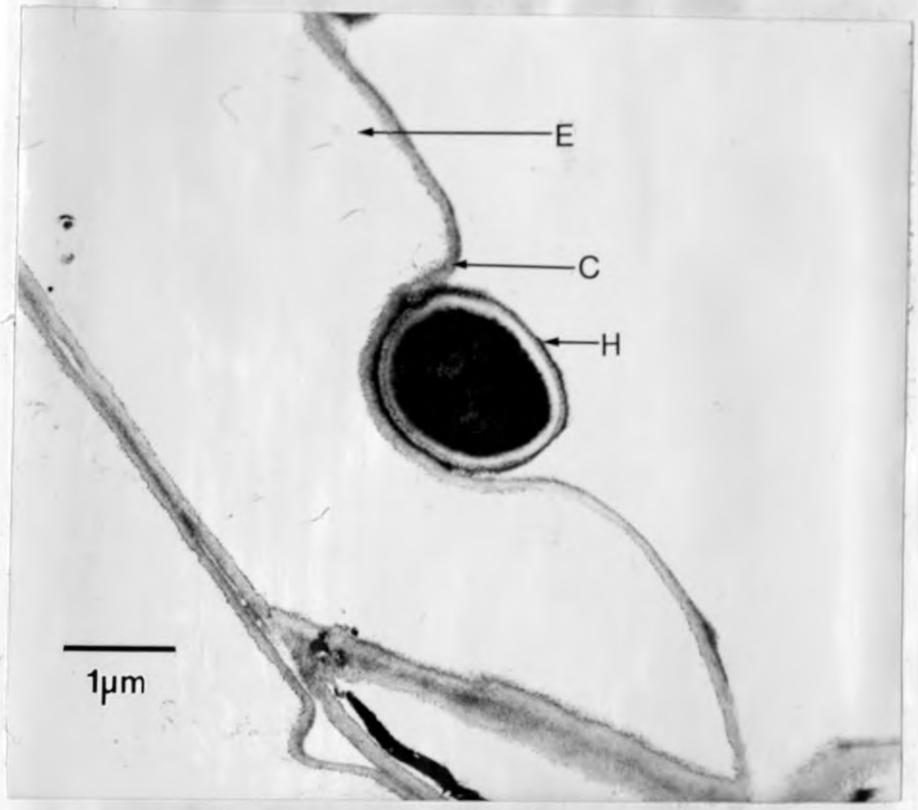

Plate 11. Transmission electron micrograph showing depression of cuticle during penetration. (Magnification X15,000)

H - hypha.

C - depressed cuticle.

E - epidermal cell.

Plate 12. Scanning electron micrograph showing synnemata (S) on cvs Rosecoco-GLP-2 (a) and M29 (b) 15 days after inoculation with isolate PG18 (Magnification X400).

CHAPTER FIVE

5.0. DISCUSSION

5.1. Identification of *Phaeoisariopsis griseola* Sacc.

The morphological characteristics of the 18 isolates of *P. griseola* conformed to those reported by Deighton (1977) who observed that conidiophores of *P. griseola* were in coremoid fasciles, densely aggregated but not adherent, straight near the bottom but slightly divergent towards the apex. He also reported that conidia were borne at the tip of conidiophores and were pale and olivaceous, straight or slightly curved with a slightly but distinctly thick hilum. In this study, good growth of *P. griseola* was obtained on bean leaf dextrose agar at a pH of 6.8 incubated at 24°C in the darkness. While sporulation was observed as early as 5 days after incubation, good sporulation was achieved after 10 days.

The conidial size and septation among the isolates of *P. griseola* were found to vary considerably but the variations were not significant. As the size increased, the number of septa also increased. This variation which reflects the probable genetic variation inherent in the fungus could enable the organism to respond differently under varying environmental conditions such as temperature, moisture, pH, and nutrients (Stakman and Harrar, 1957; Hawker, 1966). The mean conidial length, width, and number of septa obtained for the isolates of *P. griseola* used was in agreement with those reported by Saccardo (1886), Benlloch (1944), Llanos (1957), Zaumeyer and Thomas (1957), Hocking (1967), Ellis (1971), Buruchara (1983) and Karanja *et al* (1994). Deighton (1977) noted that conidia had 1-4 septa, measuring 25.5-

45µm by 4-5µm. Buruchara (1983) reported an average conidia length of 38.5µm and 6.4µm in width. He also noted that 51.5% of the conidia of *P. griseola* had 3 septa and the range was 0-7. Karanja *et al* (1994) observed that the average conidial size was 44µm long by 13.9µm wide and that most conidia of *P. griseola* had 1-5 septa, especially 2-3. The variation in conidial size and septation was wide among and within the isolates and could probably be attributed to natural variation within the pathogen and environmental conditions in areas of origin but were not found to be race-specific. The variation of these characters observed within isolates limits their usefulness as means of separating isolates into pathotypes. For example, even those isolates whose sizes and septation were not significantly different could not be grouped together because they gave different pathogenic reactions. However isolates PG5 and PG7 whose length and septation were not significantly different, gave similar pathogenic reactions and were grouped together as race 5.

The differences in mycelial growth and colouration observed in colonies of *P. griseola* isolates is a feature observed and also reported previously by Alvarez-Ayala (1979), Buruchara (1983) and Karanja *et al* (1994). Alvarez-Ayala (1979) explained this appearance to be probably due to growth of secondary mycelia arising from the normally uninhibited germination of conidia produced by the primary mycelia. On further subculturing of the isolates for subsequent studies, the growth and appearance of the mycelia was found not to be consistent for some of the isolates. For example isolates PG4 and PG15 which were initially dark appeared grey after continued subculturing. However, in some of the other colonies, for example, PG6, PG8, PG11 and PG2 the

colour was consistent even after subculturing thus implying that this feature was dependent on the particular isolate.

Buruchara (1983) observed that after two to three years of storage at 4°C and frequent subculturing (at 19°C), isolates which initially had low sporulating grey mycelial colonies produced highly sporulating dark mycelial colonies. He also noted that most colonies with the grey mycelia had low conidial harvest, but the petri-dishes containing these colonies often had condensed moisture below their covers. In the presence of high relative humidity or free moisture and under favourable temperatures, conidia of *P. griseola* readily germinate. This may be why these colonies had lower conidial harvest, a feature also noted at times on some of the dark mycelial colonies. Given this general inconsistency among some isolates, the appearance of the mycelia could probably be dependent on isolates and/or environmental conditions particularly temperature, moisture, culture medium or an interaction of these factors. This feature does not seem to be associated with virulence factors of the isolates and is thus of no significance in race typing.

Hyphal fusion was noted in Riddell slide cultures of *P. griseola* isolates and this was in agreement with results obtained by Karanja *et al* (1994) who observed that *P. griseola* frequently exhibited hyphal fusion. This phenomenon could have significant implications in its pathogenic variability. When conidia develop from heterokaryotic cells as a result of anastomosis, the segregation of disimilar nuclear in the spore encourages genetic variation. For instance in *Puccinia striformis*, anastomosis between hyphae of different genotypes gave rise to a third physiological race (Stakman *et al*, 1962). Two races of the

pathogen were inoculated on plants and when reisolation was done, a 3rd race was identified. Anastomosis has also been implicated in Benomyl torelant and sensitive strains of *Colletotrichum kahawae;* syn. *Colletotricum coffeanum* (Mwang'ombe *et al,* 1988)

All the 18 isolates collected from 15 bean growing districts were found to be highly virulent and induced characteristic ALS symptoms on cvs Rosecoco-GLP-2 and M29. Isolate PG1 did not successfully infect cv Rosecoco-GLP-2 but induced typical ALS symptoms on cv M29. These results clearly indicate that in future, cvs Rosecoco-GLP-2 and M29 should be used as universal suscept for testing the virulence of Kenyan isolates of *P. griseola*. Cultivar Rosecoco-GLP-2 was initially selected as it is known to be susceptible to ALS under field conditions while line M29 was included as a second suscept as it has been known to support some infection by ALS pathogen (Mwang'ombe *et al,* 1994).

5.2. Pathogenic variation

5.2.1. Angular leafspot symptoms on bean cultivars.

The range of symptoms induced by the *P. griseola* isolates on bean cvs was wide and this further points to pathogenic variability in the pathogen. The symptoms on the trifoliolate leaves ranged from small clearly delimited angular lesions to large coalescing lesions of greater dimensions. This variation in symptoms could neither be attributed to a particular isolate nor cultivar since different cvs responded differently to the same or different isolates. However, some isolates for example PG1 induced similar symptoms on all the cvs. Likewise some of the cultivars for example cvs Rosecoco-GLP-2, Mwitemaria-GLP-X.92,

Calima, M29 and BAT76 developed similar symptoms when inoculated with most of the isolates. The appearance of circular lesions on the primary leaves instead of angular ones as on the trifoliolates is probably due to the tenderness of the veins and veinlets of the primary leaves which cannot resist colonization by the fungus. On some of the resistant cvs, resistance was mostly expressed as a hypersensitive reaction whereas others supported some limited infection but sporulation did not occur suggesting that these cultivars are not highly resistant to ALS. Leaf chlorosis accompanied ALS symptoms on some of the susceptible cultivars such as cv E2 and Mwitemania-GLP-X.92 when inoculated with isolate PG16. However, chlorosis was also common on the resistant cultivars for instance cvs M26 and A285 when inoculated with isolates PG7 and PG10. In these cases, chlorosis and eventually leaf defoliation occurred regardless of the disease severity.

5.2.2. Race typing of *P. griseola*.

This study clearly demonstrated that isolates of *P. griseola* obtained from different bean growing districts in Kenya do exist in different races (pathotypes). However, the actual leaf area covered by lesions could not be used to separate *P. griseola* isolates into races because it only relies on the percentage infection and ignores the reaction types such as whether the lesions were sporulating or not. For example, a cultivar with a score of 5% but with non-sporulating lesions would be graded as susceptible (grade 5) if only the percentage leaf area covered by lesions was considered. However the cultivar reaction should be scored as resistant (grade 3) according to angular leaf spot

scoring scale where cultivars with non spolurating lesions are considered resistant regardless of the percentage leaf area covered with lesions. For instance, cv Montcalm inoculated with isolate PG13 had lesions covering upto 4.7% of the leaf area but no sporulation was noted and thus it was rated as resistant (grade 3).

The eighteen *P. griseola* isolates induced distinct responses in the 30 bean cvs/lines used as differentials. Even isolates of *P. griseola* from similar geographic regions varied pathogenically. For example, the isolates from Nyeri district were pathogenically different and only induced similar reactions in some but not all the cvs. Isolates from the cold areas, for example upper parts of Murang'a, Kerugoya and Nyeri districts also varied in the type of responses they induced in the differentials. Isolate PG2 from Ndeiya in Kiambu district induced susceptible reactions in all the locally grown bean cvs and lines developed at the University of Nairobi Kabete campus implying a very high adaptability to these cvs. In Kenya, there are several recommended bean cvs which include cvs Rosecoco-GLP-2, Canadian wonder-GLP-24, Mwezi moja-GLP-1004, Mwitemania-GLP-X.92, Zebra-GLP-806, Red Haricot-GLP-585, Rosecoco-GLP-288, Rosecoco-GLP-77 and Mwezi moja GLP-X-1127A. Small scale farmers also grow many types of local bean landraces. All these different types of beans provide a wide genetic pool which if matched by genes in different important pathogens would lead to physiological specialization.

Indications of field occurrence of physiological races of plant pathogens are generally based on two criteria viz: the gradual adaptation of a given pathogen to a variety earlier known to be resistant in a given area and the large differences in varietal reaction at different location or countries (Chiu *et al.*, 1965). Some of the common plant pathogens such as *Colletotrichum lindemuthianum* (Sacc. and Magn.) scrib and *Uromyces phaseoli* (Pers). Wint. on beans (*Phaseolus vulgaris* L.), *Puccinia graminis* var. *tritici* Erik. and Hen. on wheat, and *Pyricularia oryzea* on rice are a few of the many fungi which exhibit considerable degrees of pathogenic variability and have been shown to meet one or both of the above criteria (Barrus, 1911; Harter and Zaumeyer, 1941; Stakman *et al*, 1962; Goto, 1965). Wallace (1952) and Hocking (1967) reported the existence of new virulent forms of *P. griseola* attacking beans. Villegas (1954) tentatively grouped 33 single spore isolates of *P. griseola* from Columbia into 13 races on the basis of their differential reactions with 14 bean lines, while Buruchara (1983) when working with *P. griseola*, grouped 20 isolates from Columbia and one from Winsconsin U.S.A. into 7 pathotypes based on their reactions on 6 bean cvs.

The apparent high pathogenic variation observed among the Kenyan isolates of *P. griseola* was probably due to the varied number and complex gene constitution of the differential cvs used. Under such circumstances, the isolates belonging to the same race (or pathotype) when tested on a few differential cultivars may be separated when more differentials are used. Although using additional differentials gives the impression of

high variation and makes the procedure of race differentiation more complicated, the latter however becomes more exact (Kiraly, 1974). In addition the variation observed was possibly as a result of the capacity of *P. griseola* to adapt to the genetically variable bean cvs grown in Kenya. This might explain the high susceptibility of the local cvs to all the isolates as compared to the wide spread resistance of the 22 differentials obtained from CIAT.

In the past, bean cvs that have been reported to have resistance against *P. griseola* include cvs Kentucky Wonder, Alabama No.1, California small white No.643, Epicure, Cauca-27A, Caraota 260 and G4421 (Buruchara, 1983). Alvarez-Ayala and Schwartz (1979) reported that the resistance in cv Caraota 260 to *P. griseola* isolates frow Brazil was not effective against certain isolates in Columbia suggesting pathogenic variation. In the present study, some of the above cvs were susceptible to at least one or more of the Kenyan isolates of *P. griseola* tested. For example, cv Alabama No.1 was susceptible to isolates PG1, PG2, PG8 and PG15 while cv Caraota 260 was susceptible to isolates PG1, PG2, PG10 and PG18. However these cvs were resistant to some of the isolates implying that there exists a host-pathogen specialization relationship. These results were in agreement with those obtained by Alvarez-Ayala and Schwartz (1979) who observed that cvs Alabama No.1, Cauca-27A and Caraota 260 were susceptible to 1, 1 and 3 isolates respectively out of the 5 isolates of *P. griseola* they used. It was clear that the effects of resistance genes were dependent on the isolates used, a phenomenon usually observed where race specific or vertical major genes are involved (Flor, 1971; Day, 1974) and also an

indication of high degree of host-pathogen specificity between bean cvs and isolates of *P. griseola.*

Reports by different early workers showing that the above cultivars were resistant to angular leafspot may have been due to the isolates or locality they used. The methods of testing and evaluations employed by these workers may also have been responsible for the results they obtained. In identifying sources of resistance to *P. griseola,* Brock (1951), Santos-Filho *et al.* (1976) and Avila (1979) inoculated and evaluated bean plants grown in the greenhouse scoring them on the basis of the number of lesions per plant. Alvarez-Ayala (1979) compared four methods of assessing angular leafspot severity which consisted of determining the number of lesions per 4cm^2 of leaf area, lesion area, and two scales representing percentages of leaf area covered with lesions and found that all lead to the same relative rating of the host resistance and isolate virulence. On the other hand, Singh and Sharma (1975) made tests under field conditions and found `Kentucky Wonder' to be immune. It should thus be appreciated that even with very compatible host-pathogen relationship (host susceptible), ideal environmental conditions (high relative humidity and optimum temperatures) are essential prerequisites to ensure successful infection by *P. griseola* (Cardona-Alvarez and Walker, 1956, Llanos, 1957; Silvera, 1967; Alvarez-Ayala, 1979; Avila, 1979). It is thus possible to have disease escape which maybe confused for resistance if these conditions are not met. The scoring time is also an important aspect and should be constant but can be adjusted during the evaluations in a given experiment because the disease symptoms might appear early on some cultivars than on others. For example,

if the assessment is done earlier, some cultivars maybe scored as being resistant while they are actually susceptible and especially so if the isolate used has a long latent period. For example Kenyan isolates PG1 and PG6 when inoculated on differential bean cultivars induced initial susceptibility symptoms after 15 and 19 days respectively. Thus a compromise should be reached to cater for both the short and long latent periods of the pathogen on differential cultivars.

5.3 Bean tissue colonization by *Phaeoisariopsis griseola*

In this study, resistance in cultivar M26 to *P. griseola* was found to be elicited immediately on bean tissue inoculation. The pre-penetration and penetration events were similar in cvs Rosecoco-GLP-2 and M29 when inoculated with isolate PG18. This was not the case in cv M26 where conidial germination was slightly inhibited and the germtubes of the germinated conidia were shorter. Penetration in the three cultivars occurred 24 hours after inoculation and was either direct or through the stomata. The nature of the stimulus governing the direction of germtube growth and penetration was not clear because some of the germtubes were noted to have by-passed the stomata and then penetrated directly. Dickinson, (1949) suggested that the entrance through stomata maybe a positive hydrotrophic response but not all plant pathogenic fungi make such a response. Many organisms that gain ingress by direct penetration of the cuticle of a leaf produce germtubes that grow directly over stomata but never enter the leaf through stomatal openings. Two hypothesis have been proposed to explain the mechanism of direct penetration (Roberts, 1984). According to one hypothesis, the penetration peg

gains ingress by mechanical pressure while according to the other, the fungus penetrates after having exerted chemical action that partially destroys the protective covering of the plant.

Direct penetration of bean leaf by *P. griseola* appears to be through exerting mechanical pressure as indicated by the presence of an inward depression of the cell wall at the point of penetration but this does not rule out the presence of an enzymatic activity. However the fact that the cuticle surrounding the infection hyphae was not degraded after penetration suggests that any enzymatic activity was restricted only to that area adjacent to the tip of the infection hypha. The production of such a cutinolytic enzyme maybe restricted to the initial penetration of the outer part of the cuticle which contains cutin and cuticular wax.

In all the 3 cultivars, infection vesicles formed in living epidermal cells and primary and secondary hyphae developed to varying extents producing symptoms that ranged in severity from flecking to spreading lesions. The absence of rapid cell death and browning in cv M26 and the fact that development of infection vesicles occurred in this cultivar suggests that it is not highly resistant to *P. griseola*. One structural change which appears to accompany resistance in a wide range of gene-for-gene interaction is the rapid collapse and death of one or more host cells at the penetration site, linked with the death or cessation of growth of the invading fungus (Heath, 1974). O'connell and Bailey (1985) reported that extreme resistance of bean cultivars to *Colletotrichum lindemuthianum* involved rapid death and browning of penetrated epidermal cells without formation of infection vesicles or a detectable biotrophic phase. On the other hand,

extreme susceptibility involved the initial development of fungal infection vesicles within living epidermal cells followed by colonization of further host cells by intracellular primary hyphae of *C. lindemuthianum*.

The initial survival of the penetrated epidermal cells may reflect a `basic compatibility' between *P. griseola* and its host species perhaps due to a passive failure of the plant to recognize the pathogen or to the activity of other pathogen genes that regulate the invasion of living host cells. The subsequent biotrophic development of the pathogen terminated at various stages, producing the range of symptoms observed. It was short in cv M26 and prolonged in cvs Rosecoco-GLP-2 and M29. The duration of the biotrophic phase could be determined by specific recognition occurring at different times thus triggering premature host cell death and resistance. A prolonged period of biotrophic development is essential for the production of spreading lesions whereas when the biotrophic phase is of shorter duration or absent, premature death and browning of infected cells lead to restriction of both pathogen growth and extent of symptoms (O'connel and Bailey, 1985). This might explain why the colonization of tissues in cv M26 was limited as compared to that on cvs Rosecoco-GLP-2 and M29. A quite different impedment to successful infection in the resistant cultivar may be the absence or scarcity in the host of nutrients essential for development of the pathogen. Keitt and Boone (1954) isolated mutants in the apple scab fungus *V. inequalis* whose requirements for vitamins, nitrogen bases, or amino acids were specific. Pathogenicity was lost in some of the mutants unless the particular requirement was supplied with the inoculum.

Tissue colonization by *P. griseola* was more extensive in cvs Rosecoco-GLP-2 and M29 than in cv M26. In all the cultivars, colonization was confined to the lower epidermal and spongy mesophyll cells and the appearance of lesions on the upper surface of the leaf might probably be as a result of diffusible fungal toxins or enzymes released from the infected cells. Furthermore, the killing of the uninfected cells at the margin of the spreading lesions in cvs Rosecoco-GLP-2 and M29 was closely associated with cell wall dissolution probably due to fungal toxin of enzyme activity. This is further supported by the fact that the margin of the lesion was slightly in advance of the tips of the ramifying hyphae. The hyphae which colonized the tissues was closely and conspicously septed as compared to that in culture and this points out that probably the growth of *P. griseola* on artificial media is not the same as under natural conditions. This conspicous septation in tissue maybe a survival mechanism for the fungus since hyphal septa are known to act as the first line of defence against damage and that septate hyphae survive drying much better than do aseptate hyphae (Smith *et al*, 1978).

The rate and time of sporulation was found to be an important distinguishing feature in the reactions of the 3 cultivars to *P. griseola*. In cv Rosecoco-GLP-2, spore production was abundant and occurred 8 days after inoculation but in cv M29, it was reduced and occurred 4 days later whereas in cv M26, it was inhibited. The initial stagnation of events and the suppression of sporulation on the resistant and moderately resistant cultivars noted here is a feature also reported in other host-pathogen relationships, for example in resistance of

Coffee arabica to *C. coffeanum* Noack (Mwang'ombe and Shanker, 1994).

The restriction of colonization and sporulation in the two cultivars may have been as a result of some physical or chemical barriers. Although a pathogen may gain easy entry into the host, existing structural barriers within the tissues may contribute to resistance by limiting the pathogens' subsequent development. Such barriers may be generally distributed and reduce the pathogen rate of colonization, be localized at particular impenetratable tissues and confine the pathogen to specific areas or be features of the outer layers and restrict its sporulation. Alternatively, chemical changes within the infected tissues which are harmful to the pathogen may occur, for example, certain inhibitory substances like phytoalexins are formed or activated only when the pathogen comes into contact with the host cells (Allen, 1959).

From the results obtained in this study, it is clear that resistance in cultivar M26 to *P. griseola* is due to limited conidial germination and an inhibition in tissue colonization and sporulation, whereas the intermediate reaction in cv M29 is due to limited and delayed sporulation. It is also evident that the rate-reducing resistance against *P. griseola* reported by Buruchara (1983) is operational in these cultivars. The onset of tissue colonization two or three days later in the resistant cultivar and the delayed sporulation in the intermediate cultivar may be attributed to either long latent periods or race-specific resistance in these cultivars.

The specific pathogen-cultivar responses shown by *Phaseolus vulgaris* L. after inoculation with *P. griseola* are thought to be controlled by a gene-for-gene relationship between the pathogen and its host (Mulindwa, 1980). The gene-for-gene hypothesis has been interpreted to imply that complementary genes of the plant and the pathogen control a yes: no interaction (Van der Plank, 1975). Such an interaction would be expected to produce clearly distinguishable phenotypes; for example, completely distinct susceptible and resistant reactions. However, it is clear from the results reported here and from some other studies (e.g. by Stakman and Harrar, 1957) that the final phenotypes of race-cultivar interactions cannot always be assigned satisfactorily to only two categories as many of the symptoms produced are intermediate between extreme resistance and susceptibility. Thus the existence of intermediate reactions must be accomodated in any model of the molecular basis of cultivar specificity.

CONCLUSION AND RECOMMENDATION.

Due to the distinct differences in responses of the differential cultivars to the different isolates, this study established that physiological specialization exists in *P. griseola* isolates. However the apparent high pathogenic variation observed among the isolates was probably due to the varied number and complex gene constitution of the differential cultivars used. Due to the high pathogenic variability in *P. griseola*, breeding programmes have to aim at developing bean cultivars with race non-specific resistance and all efforts in control of angular leafspot disease should be geared towards this aspect. Alternatively, bean cultivars resistant to particular races can be selected and grown in areas where these races occur but this can only be a short term solution because new pathogenic races might arise or be introduced in a geographic region.

The studies conducted on bean tissue colonization by *P.griseola* revealed that resistance to angular leafspot is not complete and involves either inhibition of germination, colonization or sporulation. However, it was not clear whether the mechanism of resistance in the resistant cultivar is mechanical or chemical and more research work should be carried out to give more light to this aspect which can then be used in breeding for resistance. Likewise, cytochemical studies should be carried out to determine the cause of cell death in the resistant cultivar as well as the cause of lesions which develop on the upper surface of the leaf where colonization does not occur.

Any differences in structural and morphological characters in resistant and susceptible bean cultivars should also be established. Although it is widely viewed that structural

elements are of little real consequence and that resistance is based largely on physiochemical interactions within the host system, there are good reasons to suspect that mechanism based solely on plant structure can contribute usefully to the overall disease resistance. Thus, there is justification for renewed efforts to explore structural mechanisms to assess their value and then perhaps to exploit them in bean breeding programmes.

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APPENDICES

APPENDIX 1. Anova for the variation of spore length of 10 isolates.

Source	SS	df	MS	F-value
Main Effects				
Isolate	164.39	9	18.27	0.99 ns
Error	562.03	30	18.73	
Total	726.42	39		

ns = not significant at 5% level.

APPENDIX 2. Anova for the variation of spore width of 10 isolates.

Source	SS	df	MS	F-value
Main Effects				
Isolate	38.80	9	4.31	3.50**
Error	36.31	30	1.23	
Total	75.39[9]	39		

** = significant at 5% level.

APPENDIX 3. Anova for the variation in spore septation of 10 isolates.

Source	SS	df	MS	F-value
Main Effects				
Isolate	10.1	9	1.12	0.90 ns
Error	37.5	30	1.25	
Total	47.6	39		

ns =not significant at 5% level.

APPENDIX 4. Anova for number of synnemata per lesion in cultivars Rosecoco-GLP-2, M29 and M26.

Source	SS	df	MS	F-value
Main Effects				
CULT	9668.9	2	4834.4	199.3***
Error	585.2	27	21.7	
Total	9254	29		

*** = significant at 0.1%

APPENDIX 5. Anova for the number of conidiophores per synnema in cultivars Rosecoco-GLP-2, M29 and M26.

Source	SS	df	MS	F-value
Main Effects				
CULT	25798.2	2	12899.1	82.3***
Error	433	27	156.8	
Total	3003.1	29		

*** = significant at 0.1%.

Appendix 6. Anova for the differential reactions of the 18 isolates of *P. griseola* on 30 bean cultivars and their interactions.

Source	SS	df	MS	F-value
Main effects				
Cultivar (C)	82386.5	29	2840.9	138.4***
Isolate (I)	12360.9	17	727.1	35.4***
Interaction				
C X I	90233.8	493	183.0	8.9***
Error	22172.9	1080	20.5	
Total	207154.1	1619		

Significant at 0.1% level.