# RESISTANCE TO, AND EXTENT OF HYPHAL COLONIZATION AND SPORULATION OF *Mycovellosiella cajani* IN PIGEONPEA LINES

**BY**



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## DECLARATION

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

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# DEDICATION

To my husband Samson J. Muriungi for his love and guidance.

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## LIST OF ABBREVIATIONS

DDSA - Dodecanyl Succinic Anhydrite

BDMA- Benzyl Dimethyamine

pipes - Piperazine - $N_1$ $N^1$ - bis (2 ethanosulfonic acid)

*M* - *Mycovellosiella*

*C* - *Cercospora*

SEM - Scanning Electron Microscopy

PLDA - Pigeonpea Leaf Decoction Agar

NGT - Non Gradular Trichome

ICRISAT-International crop research institute for semi arid tropics

*P - Phaseoliopsis*

*C. coffeanum*- *Colletotrichum coffeanum*

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## ABSTRACT

Pigeonpea (*Cajanus cajan*) in Kenya is attacked by *Mycovellosiella* leafspot, which is a highly devastating disease. Pathogenicity of *Mycovellosiella cajani* on susceptible pigeonpea was investigated under glasshouse conditions to provide proof for pathogenicity, which showed that the isolated fungus is pathogenic to pigeonpea.

Fifty pigeonpea accessions planted at Kabete and Katumani were assessed for reaction to *M. cajani*. The same accessions were also evaluated for resistance/ susceptibility to *Mycovellosiella* leafspot at seedling and flowering stage in the glasshouse. Rates of disease increase in infected leaves and defoliation varied among the pigeonpea accessions studied. The most susceptible accessions to *M. cajani* were MKS TK 115, MKS KO 161/1, MKS KB 94/1 and MKS KO 252. Accessions KZ 56, ICEAP 00753, ICPL 93015, ICPL 86091, MKS KO 31 and KO 31 were resistant to *Mycovellosiella* leafspot disease.

Resistant, ( KZ 56, ICPL 87109, ICEAP 00554), Intermediate (NPP 670, ICPL 93015, MKN KO 74) and susceptible (MKS KO 161/1, MKS KO 115, MKS KO 252) pigeonpea plants were inoculated in the glasshouse to study histological reactions. Within 3 hours from inoculation, spores of the fungus germinated in both susceptible and intermediate susceptible pigeonpea plants. Spores never germinated until 8 hours after inoculation in resistant varieties. Resistant varieties exhibited cell collapse in mesophyll cells directly below the penetration site and in surrounding tissue in 24 hours after inoculation and hyphae was limited to the epidermal cell penetrated first. Resistant varieties seemed to produce stomatal exudates that restricted fungal ingress.

Non gradular trichome seemed to block fungal penetration, both in resistant, intermediate resistant and susceptible genotypes.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Origin, production and utilization of pigeonpea

The pigeonpea (*Cajanus cajan* (L) Millsp) is believed to have originated in South Asia and to have spread to Africa some 4000 years ago (Van de Maesen, 1980). It probably spread from Africa to other Caribbean region about 500 years ago and is now found in most parts of the American tropics. The crop is also grown in South East Asia and, to a limited extent in East Asia and Australia.

Almost 90% of the world's pigeonpeas are grown as a multipurpose legume crop. In East Africa, it is grown on a large scale in Kenya, Tanzania and Uganda, with smaller areas of the crop found in Ethiopia and Burundi (Silim, *et al.*, 1993). Pigeonpea yield however, has remained low with an average of only 300-700 kg/ha (Nene and Sheila, 1990).

Pigeonpea is the most important grain legume in the semi-arid region of Eastern Kenya. Pigeonpea is produced over an area of 200,000 hectares, mainly in Machakos, Kitui, Embu, Tharaka Nithi and Makueni Districts. Productivity is highly variable, depending on the cropping system and range from 500 to 800 kg/ha. It is mainly consumed as whole dry grain in a number of dishes, but a large amount is also consumed in form of greenpeas (Allain le Roi, 1997; Holkar *et al.*, 1991)

Pigeonpea is primarily grown for dry grain. These are usually boiled and mixed with maize or fried and eaten as a vegetable. The crop is often grown as boundary plants, hedges or windbreaks, while the woody stems are used as fuel wood and for roofing and making baskets.

In India, the dry grains are split and made into 'dhal' (Khan and Rachie, 1972). The dried husks and rejected grain can be compounded with other feed and fed to cattle, poultry and pigs (Onim, 1982). Pigeonpea is also used as green manure, cover crop and for control of soil erosion.

The role of pigeonpea and other pulses in human and animal nutrition is largely that of supplying protein. Protein content of dry grains of pigeonpea is in the range of 17.5-28% with a mean of 20.9% (Aykroy and Doughty, 1964). Green leaves of pigeonpea contain 21.06-mg/kg crude protein that is essential for nutrition of sheep and goats (Ikhimioya and Olagunju, 1996).

### 1.2 Diseases of pigeonpea

Among the diseases of pigeonpea, *Fusarium* wilt caused by *Fusarium udum* and *Mycovellosiella* leafspot caused by *Mycovellosiella cajani* are known to cause yield losses that may be as high as 75 - 85%, depending on the location and season (Reddy *et al*., 1993).

### 1.3 *Mycovellosiella* leafspot

*Mycovellosiella* leafspot caused by *Mycovellosiella cajani* (Henna) Rangel ex Trotter (Syn. *Cercospora cajani* Henn *Vellosiella cajani* (Rangel) is the most severe leafspot disease of pigeonpea in areas with high rainfall or during wet seasons in the drier areas (Muller, 1950; Khan and Rachie, 1972). It spreads rapidly during a wet growing season and has been reported as one of the most important diseases of pigeonpea in East Africa (Njoya, 1991). Although *Mycovellosiella* leafspot can be controlled by use of chemicals, the high cost of fungicides prohibits their use especially by the low-income subsistence farmers.

Development of resistant varieties may be the most reliable and cost effective method of controlling *Mycovellosiella* leafspot .

### 1.4 **Breeding for disease resistance in Pigeonpea**

Breeding for resistance to disease has been a major objective for pigeonpea breeding programme in Kenya. However, much of the effort has been devoted to identification of resistant genotypes to *Fusarium* wilt (Njoya, 1991). In recent years, incidence of leafspot damage has increased considerably especially during wetter seasons in pigeonpea growing areas. Low resistance to this disease has been noted in cultivars released recently or the new early maturing breeding lines.

Determination of resistance to foliar diseases in crops can be achieved through various procedures such as assessment of disease incidence and severity in specified plant populations (James, 1974). The study of disease progress using areas under disease progress curves (Johnson and Beute, 1986) or apparent rates of disease progress (Krantz, 1974; Johnson and Beute, 1986) can also serve the same purpose. Some workers have also screened for resistance to *Cercospora* leafspots in several pathosystems, groundnuts (Hemingway, 1954; Garren and Jackson, 1973) using various components of plant reaction to disease. These include percent spore germination on inoculated leaf surface, incubation period, lesion size or density per unit of time, rate of lesion expansion or multiplication and latent period (Njoya, 1991).

Other components of resistance to *Cercospora* species are percentage of sporulating lesions after specified periods of incubation, quantity of conidia on sporulating lesions per unit time or lesion area and time to total necrosis of infected leaf (Melouk and Banks, 1978; Nevill, 1981; Gobina *et al.*, 1983; Shew *et al.*, 1989).

This study was designed to investigate the reaction of pigeonpea germplasm to *Mycovellosiella* leafspots caused by *Mycovellosiella cajani*, as well as assessment of hyphal colonization and sporulation of *Mycovellosiella cajani* in pigeonpea lines.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Pigeonpea

### 2.1.1 Taxonomic history

*Cajanus* as De Candolle founded a genus in 1813, based on two species, *C. flavus* and *C. bicolor* (Thothathri and Jain, 1980). De Candolle attributed both species to India, with a note stating that *C. flavus* is also cultivated in America. He cited *Cytisus cajan* (1753) under *Cajanus flavus*, thereby making it clear that the Linnaean plant is the sama as his *Cajanus flavus*. This type species of the genus is *Cajanus cajan* (L) Millsp, based on *Cytisus cajan* (L).

### 2.1.2. Origin of pigeonpea

Pigeonpea (*Cajanus cajan*) (L) Millsp. ) Is a drought tolerant legume widely grown in the tropics. Its origin is controversial and has interested many scientists. Some who favour Indian origin (Zeven and Zhukousky, 1975; Van de Maesen, 1980), mentioned pigeonpea, the plant on which the genus was founded, as native to the (East) Indies (including Srilanka). Van de Maesen (1980) suggested that it was native to Malaysia. Sturtevant (1972) mentioned that Schweinforth during his travels in Africa between 1868 - 1871 reported the presence of a seed of pigeonpea in Egyptian tombs of the 12th dynasty (2200 - 2400BC). Other workers (Good, 1964; Purseglove, 1968; Zeven and Zhukovsky, 1975) are of the opinion that *Cajanus* is probably native to Africa, from where it spread to India as a secondary center. Westphal (1960) noted the absence of pigeonpea occurring wild in India and to several finds of wild pigeonpea in Africa.

## 2.2 Cercospora leafspot

### 2.2.1 History and geographical distribution

More than 2000 species names have been attributed to *Cercospora*. Johnson and Valleau isolated *Cercospora* from 28 different host plants in 16 families (Ellis, 1971) Morphologically and culturally, they all appeared to belong to a single species. *Cercospora* species are often weak parasites on dead, drying or physiologically diseased plant tissues with occasional serious injury to healthy plants.

*Cercospora* leafspots are widely distributed and have been reported in Africa, North and South America, Asia and Europe. Chupp (1953) listed *Cercospora* leafspot on 148 different plant families and on 60 hosts in Kenya (Natrass, 1961; Ondiek, 1973) where Muller first reported it in 1950.

### 2.2.2 Economic importance of *Cercospora* leafspot

Crop damage attributed to *Cercospora* species attack is both direct and indirect. They extend from the field to storage (Hemingway, 1954; Garry and Ruppel, 1971; Hilty *et al.*, 1979). On groundnuts, early leafspot caused by *Cercospora arachidicola* and late leafspot caused by *Cercosporidium personatum* occur wherever groundnuts are grown and can cause yield losses of 50% or more (Garren and Jackson, 1973) The duration of growth is determined by defoliation resulting in premature death of the crop (Hemingway, 1954). On cowpeas, two species of *Cercospora*, *C. canescens* and *C. cruenta*, can cause severe spotting and defoliation (Schneider and Sinclair, 1975). On maize, *Cercospora* leafspot has caused losses as great as 20% in Tennessee (Hilty *et al.*, 1979) with up to 100% lodging (Rupe *et al.*, 1982).

Limited work has been done to establish the losses caused by *Mycovellosiella* leafspot on pigeonpea in Eastern Africa. Study done in Makerere University, Kampala, Uganda showed that the disease can reduce grain yield by up to 85% (Onim, 1980). These results supported observations made by Khan and Rachie (1972) and Rubaihaiyo and Onim (1975), who indicated that *Mycovellosiella* leafspot was an important disease of pigeonpea in Eastern Africa.

### 2.2.3 Symptomatology of *Cercospora* leafspots

Leaf spots varying in distinctness from a faint discolouration to characteristically masked necrotic lesions are typically of *Cercospora* leaf spots (Chupp, 1953). These turn ashy grey if environmental conditions favour sporulation.

Lesion shapes vary from irregular, circular, elliptical to rectangular as dictated by leaf venation. Rectangular lesions measure 0.5 to 1.0 by 2.5 cm, whereas others have a diameter of 2.7-mm (Hemingway, 1954; Baxter, 1956; Latch and Hanson, 1962; Kingsland, 1963).

Coalescence produce aggregate lesions of greater dimensions and in maize and cassava, severe leaf blight has been reported (Kingsland, 1963; Teri *et al.*, 1980). With severe attack, dying portions get dehydrated and shrink away from the living tissues leaving a shot-hole effect. Complete defoliation is caused by virulent species. Many *Cercospora* species also infect the blossoms, fruits, pods, succulent petioles and young stems (Chupp, 1953). The foliar symptoms typically develop later in the growth of the crop often after flowering as reported for the leaf spot of cowpea (Fery *et al.*, 1977; Schneider and Sinclair, 1975; Verma and Patel, 1969;), mungbean (Mew *et al.*, 1975), groundnut (Hemingway, 1955; Fowler, 1970) and maize (Kingsland, 1963).

*Cercospora* infections on pigeonpeas first appear on the under surface of leaves as small light brown spots of 1-2 mm in diameter (Singh, 1932). These spots are round at first but later become irregular in outline and occasionally several of these coalesce forming irregular areas as large as 15 mm x 15 mm. Spots seldom cross the midrib of the primary veins of the leaflets. The centre of these spots is dark brown and bears the fascule of conidiophores with conidia. On older spots where conidiosphores have ceased to form spores, infected areas become thin and translucent. In advanced stages the whole leaf dries, curls and ultimately falls (Singh, 1932).

On pigeonpea, *Mycovellosiella* leaf spot is characterised by brown leaf spot, sub-orbicular, usually indefinite 1-3 mm wide, but sometimes definite sub-orbicular measuring as wide as 1 cm and , often with the lesion becoming grey in the centre when older (Deighton, 1974).

**2.2.4 Etiology**

**2.2.4.1 Classification of the fungus**

*Mycovellosiella cajani* (Henn) Rangel ex Trotter Syn *Cercospora cajani* Henn = *Vellosiella cajani* (Rangel) is the causal agent of *Mycovellosiella* leafspot of pigeonpea (Deighton, 1974). It is grouped together with the form genus *Cercospora* that is placed in the form family Dematiaceae and in the form-order moniliales of the form-class Deuteromycetes and subdivision Deuteromycotina (Alexopolous and Minns, 1979).

2.2.5 **Morphological characteristics of the pathogen**

Morphological characteristics of the conidia and conidiophore provide the main taxonomic criteria for species delimitation (Hughes, 1953). Conidiophores of *Mycovellosiella cajani* are

terminal or arising at lateral branches on the external mycelial hyphae, pale or rather pale olivaceous, 5-35μ long, 4-7μ wide. The distinctive characters of *Mycovellosiella* are thickened conidial scars and the production of an assurgent secondary external mycelium on the hyphae of which the conidiophores are borne terminally and as lateral branches (Deighton, 1974). Conidial scars are about 1μ in diameter. Conidia varying from almost colourless to moderately pale olivaceous, catenate, sub cylindric, with shorter straight ones while the longer ones are slightly curved, 0-9 septation and 10-129 x 3-6μ (10-35μ in var. *cajani*). Three varieties of *M. cajani* var. *cajani*, *M. cajani* var. *indica*, and *M. cajani* var. *trichophila* have been distinguished (Deighton, 1974).

**2.2.6 Cultural characteristics of *Cercospora* species**

Extensive work has been carried out worldwide on a number of *Cercospora* species. Germtubes emerge from any of the conidial cells within 4 hours (Jenkins, 1938; Alderman and Beute, 1986; Cooperman and Jenkins, 1986).

In 1969, Sobers noted that on Potato Dextrose Agar (PDA), the genus is characterised by gray colonies with or without a pink pigment. Sporulation is not easy to achieve and some workers have reported on the fastidious requirement for *in vitro* sporulation (Nagel, 1934; Diachun and Valleau, 1941; Cooder and Brown, 1970; Smith, 1971). Owing to this, it has been common practice to subject a few isolates of a given species to various cultural manipulations. This involves such variables as light, nutrition and temperature (Berger and Hanson 1963b; Calpouzous and Stallknecht, 1967; Miller, 1969;Vathakos and Walters, 1979; El-Gholt *et al.*, 1982). The optimum temperature for conidial germination of *Mycovellosiella cajani* is 25°C and optimum pH for sporulation is 5 (Njoya, 1991).

2.2.6.1. **Growth and sporulation on culture media**

As early as 1934, Nagel found that the nature of the nutrient substratum has a strong bearing on conidial production. Nagel, 1934 further observed that a medium suitable to a particular species for optimal sporulation might not be satisfactory for other species of *Cercospora*.

Profuse sporulation of various *Cercospora* species on their respective host decoction agar is known to occur 7-12 days from time of seeding by a series of workers. This has been observed on *C. nicotianae*, *C. beticola*, *C. zebrina* and *C. arachidicola* (Diachun and Valleau 1941; Berger and Hanson, 1963a; Calpouzous and Stallknecht, 1965; Smith, 1971; El-Gholt *et al.*, 1982). Despite this, Vathakos and Walters (1979) and Cooperman and Jenkins (1986) have reported erratic sporulation on some host-based media while working with *C. asparagi* and *C. kikuchii*, respectively. The latter pair observed abundant conidiophore formation and extremely sparse sporulation. Njoya, (1991) reported maximum sporulation 14 days after plate inoculation with *M. cajani* on pigeonpea leaf decoction agar.

Carrot leaf decoction agar is yet another medium that has proved useful in induction of sporulation of *Cercospora* species. Abundant sporulation on the carrot medium has been attained with *C. asparagi*, *C. kikuchii*, *C. zebrina* and *C. devisii* (Kilpatrick and Johnson, 1956; Latch and Hanson, 1962; Berger and Hanson, 1963b; Cooperman and Jenkins, 1986). PDA is a common medium for culturing fungi but success in inducing sporulation of *Cercospora* species has been reported only in a few cases (Nagel, 1934; Latch and Hanson, 1962; Sobers, 1969).

In 1965, Calpouzous and Stallknecht, not only emphasized the importance of selection of appropriate medium but also the interaction between light and temperature for maximum

sporulation. Continuous darkness has been found as the optimum condition for sporulation of *C. nicotianae*, *C. devisii* and *C. kikuchii* (Vathakos and Walter, 1979). Alternative dark and light period favours sporulation of *C. zebrina*, *C. asparagi* and *C. kikuchii* (Kilpatrick and Johnson, 1956; Berger and Hanson, 1963b; Cooperman and Jenkins, 1986). There are yet other *Cercospora* species, which require exposure to continuous light for sporulation to occur. This is the case with *C. beticola* as reported by Calpouzous and Stallknecht (1967).

The pH of the medium influences sporulation in fungi. Growth of *Cercospora* species has been found to be favoured by acidic media (Njoya, 1991). Singh (1932) reported that medium adjusted to pH 6.7 supported optimum growth of *C. indica*. The fungus rendered the medium in which it grew acidic. Maximum growth for *C. zebrina* has been reported to be at pH 5.2 (Berger and Hanson, 1963b). The optimum pH for *Mycovellosiella cajani* has been reported to be pH 5 (Njoya, 1991).

*Cercospora* species grow and sporulate within a temperature range of 15-30°C. The optimum for growth and sporulation reported for *C. zebrina*, *C. asparagi* and *C. davisii* is 24°C while for *C. gossypina*, *C. beticola* and *C. nicotianae* it is 21-29°C, 15°C and 26°C, respectively (Calpouzous and Stallknetch 1967; Stavely and Nimmo, 1968; Miller, 1969; Cooperman and Jenkins, 1986).

2.2.6.2 **Factors affecting conidial germination**

(i) **Time**

The ability to produce colonies and sporulate in culture was increased with successive transfer of pure sporulating conidia (Wagara, 1996). Subculturing, a method of transferring densely sporulating areas, has induced sporulation in species of *Cercospora* (Nagel, 1934;

Calpouzous, 1954; Jones, 1958; Calpouzous and Stallknecht, 1965). Cooperman and Jenkins (1986) reported that germination of conidia occurred in cultures of *C. asparagi* after 6 days of plate inoculation thus decreasing the number of viable conidia. Njeru (1988) reported that sporulation of *C. mollucellae* increased to a peak and then declined during the time of incubation.

Infection through artificial incubation of host plants has been accomplished at ages between two weeks and ten months using varying conidial concentrations. On leaf surfaces, conidia germinate from one or more cells within 3 - 6 hours (Jenkins, 1938; Baxter, 1956; Berger and Hanson, 1963b). The incubation period ranges from 6 - 22 days depending on the isolate, inoculum concentration, host plant and prevailing temperature (Latch and Hanson, 1962; Berger and Hanson, 1963b; Cooperman and Jenkins, 1986). Under favourable conditions, the pathogen sporulates on infected plant tissues.

(ii)**Temperature**

*Cercospora* species have been shown to germinate at a wide range of temperatures and in the presence or absence of light (Njoya, 1991). The optimum temperature for conidial germination reported for *C. zebrina, C. asparagi, C. davisii* is 24°C while for *C. eruenta* is 25°C (Nagel, 1934). The optimum temperature for conidial germination reported for *M. cajani* was 25°C (Njoya, 1991).

(iii) **Moisture.**

Conidial germination of *Cercospora* species is inhibited by the presence of free water case of (Judd and Peterson, 1972). Such species as *C. musae* and *C. cruenta,* however require a film of water in order to germinate (Meredith, 1970; Ekpo and Esuruoso, 1977). Ekpo and

Esuruoso (1977) found that leaf extract of cowpea enhanced spore germination in *C. cruenta*. Conidia of *C. asparagi* germinated readily in free water both on the leaf surface and on the glass slides (Cooperman and Jenkins, 1986).

### 2.2.7. **Epidemiology of *Cercospora* leafspot**

*Cercospora* leafspot severity on various hosts can be attributed to various factors. The increase in severity in maize of gray leafspot, caused by *Cercospora zeae-maydis*, has been attributed to increases in area planted to reduced tillage and no tillage practices (Latterell, and Rossi, 1983). *C. zeae-maydis* is wind-dispersed, and if the neighboring fields are heavily infested with the pathogen, the residue may re-introduce the pathogen in the field (de Nazareno *et al.*, 1990). Sustained periods of high relative humidity are more important for the development of *C. zeae-maydis* than plant and leaf age (Beckman and Payne 1982).

During the growing season, sporulation of *Cercospora* is more abundant on those lesions under warm, foggy or humid conditions on maize (de Nazareno *et al.*, 1992). Apical progression of *Cercospora zeae-maydis* on maize from lower leaves late in the season suggests that host plant maturity and leaf age affect disease severity (Beckman and Payne, 1982). Also in pigeonpea *Cercospora* leafspot progression from lower leaves occurs during the flowering period (Njoya, 1991).

Plant disease severity also depends on plant density. de Nazareno *et al.*, (1991) reported highest gray leafspot severity in maize when plant density is low Gene activity for the chlorotic lesion response also may be influenced by light quantity or intensity because light affects *Cercosporin* production of *Cer*cospora (Jenns *et al.*, 1989).

Although the effect of tillage on plant disease epidemiology are complex, Boosalis and co-workers (1986) stated that the degree of influence on plant diseases by crop residues is generally related to the amount of residues remaining after planting.

Abundant moisture, air temperature and host susceptibility in addition to the presence of a source of inoculum on the soil are necessary for *Cercospora* to cause an epidemic (de Nazareno *et al.*, 1993). *Cercospora* leafspot progress and severity are influenced by lesion type (Freppon *et al.*, 1996) and temperature affect lesion expression by influencing a change in lesion type from resistant to susceptible overtime (Thakur *et al.*, 1989). Rathaiah, (1977) reported that germinated spores of *Cercospora beticola* are capable of withstanding several diurnal cycles on leaves and still penetrate the host.

**2.2.8 Screening pigeonpea genotypes for resistance to *Mycovellosiella cajani***

Numerous disease resistance-screening techniques have been developed for evaluation of legumes under controlled conditions, including laboratory and greenhouse methods. Such systems of evaluation have the advantage that they tend to be rapid and can usually be employed throughout the year without reference to season. For effective screening of resistance against *Mycovellosiella* leafspot and to be able to develop laboratory and greenhouse resistance- screening techniques that can accurately predict resistance in the field, information about the effect of plant age and leaf position on the susceptibility of pigeonpea plant to *Mycovellosiella* leafspot is necessary. Artificial epiphytotics are also necessary to permit selection for resistant types (Drolsom and Dickson, 1954).

Selection for resistance to *Mycovellosiella cajani* in the field has been reported (Onim and Rubaihayo, 1976, Rodriguez and Melendez, 1984). There is evidence that field resistance

tend to be confounded by variations in host development, perhaps leading to spurious conclusion in the identification of resistance to *Cercospora* leafspot (Nevill and Evans, 1980). Njoya, (1991) reported that all the eight pigeonpea genotypes inoculated in greenhouse were susceptible to *M. Cajani* and in 12-14 days after inoculation, *Mycovellosiella* leafspot was noted in some inoculated leaflets.

Khan and Rachie (1972) reported that there was a wide variation in resistance pigeonpea Lines in their resistance to *M. Cajani*. Onim and Rubaihayo (1976) also observed such situation while screening pigeonpea cultivar resistance to *M. Cajani*. They suggested that resistance to *M. Cajani* might be polygenic.

Differences in partial resistance of early leafspot have been reported in groundnuts.Some genotypes developed fewer lesions per leaf (Sowell *et al.*, 1976; Hassan and Beute, 1977). Reduced sporulating lesion (Foster *et al.*, 1980) and percent of lesion sporulation (Ricker *et al.*, 1985) as well as increased latent period (Nevill, 1981) and increased time of defoliation (Ricker *et al.*, 1985) have been reported in other host - pathogen systems.

2.2.9. **Histopathological relationship of plant pathogens on their respective host plants.**

At high humidity and temperatures between 16 and 30°C, conidia of many *Cercospora* species germinate within 3 to 6 hours to give one or more germtubes on the leaf surface of their hosts such as alfalfa (*Medicago sativa*) (Baxter, 1956) and groundnut (Jenkins, 1938; Abdu *et al.*, 1974; Alderman and Beute, 1986). Penetration of *Cercospora* species into host tissue usually occurs 24 to 48 hours after inoculation and is often via the stomata (Baxter, 1956; Latch and Hanson, 1962; Beckman and Payne, 1981). Generally, no appressoria are formed prior to penetration but in some cases, appressoria formation has been observed on

inoculated leaf surface of some plants such as sugarbeet (Solel and Minz, 1971), maize (Thorson and Martison, 1989) and groundnut (Melouk and Aboshosha, 1989). Solel and Minz (1971) suggested that penetration is a hydrotropic response.

The period between inoculation and symptoms expression ranges from 6 to 22 days and is dependent on ambient temperature and humidity conditions, inoculum density, form and virulence, and hosts stage of development and inherent susceptibility (Chowdhury, 1944; Cooperman and Jenkins, 1986 Latch and Hanson, 1962; Berger and Hanson, 1963a; Gobina *et al*, 1983). The lesions produced on the infected tissues are due to necrosis on the invaded plant cells (Chupp, 1953; Solel and Minz, 1971). Important to lesion development is the secretion of a red toxin, *Cercosporin* by the invading fungal mycelium (Daub, 1982). On illumination, *Cercosporin* generates free radicals that damage cell membranes thereby causing cell death (Daub, 1982).

Leaf lesions due to *Cercospora* species usually vary in distinctness from faint discoloration to marked necrosis of the infected area (Chupp, 1953). The leaf spots are usually pale to dark - brown with raised margins of darker shade (Ellis, 1976). The lesions may also assume angular, ellipsoidal, round or irregular shape but they are often delimited by leaf veinlets. The size of the lesions usually ranges from 0.5 to 10 mm (Hemingway, 1954, Baxter, 1956, Kingsland, 1963; Deighton, 1974), but the necrotic spots may coalesce to give a blighted leaf appearance under severe infections (Kingsland, 1963; Teri *et al.*, 1980). Premature defoliation of the infected plants could also occur under heavy infection (Elston *et al.*, 1976). White leafspot of sesame caused by *C. sesami* is characterised by 0.5 to 5 mm diameter, sub-round, white or yellowish - brown centered, dark to purple lesions that are often scattered on both surfaces of the leaf during the early stages of the diseases (Nyanapah, 1992). The lesions

later enlarge and may coalesce to give extensive angular or irregular, concentrically connate necrotic regions of up to 40 mm diameter.

Direct penetration of bean leaf by *Phaeoisariopsis griseola* was reported to be due to mechanical pressure (Wagara, 1996) as indicated by the presence of an inward depression of the cell wall at the point of penetration. This does not rule out the presence of enzymatic activity. Rapid death and browning of penetrated epidermal cells without formation of infected vesicles or a detectable biotrophic phase have been found in *Colletotricum lindemuthianum* (O' Connell *et al*, 1984).

Development of infection vesicles in beans infected by *P.griseola* indicated that the cultivars are not highly resistant to the pathogen (Wagara, 1996). The initial survival of the penetrated epidermal cell may reflect basic compatibility between pathogen and its host species perhaps due to a failure of the plant to recognize the pathogen or due to the activity of other pathogen genes that regulate the invasion of the living host cells (Wagara, 1996).

Sporulation of *Cercospora* species on host surface usually occurs within 21 days following inoculation and is dependent on light, temperature, host susceptibility and site of infection (Berger and Hanson, 1963a, Nevill, 1981). During sporulation, conidiophores arise from beneath the epidermis in the substomatal chamber or between the guard cells. As the conidiophores enlarge, guard cells are forced apart and this usually results into ruptured epidermis. Conidiophores may also emerge through the stomata and are usually branched and intertwined forming ropes on the leaf surface (Berger and Hanson, 1963a, Beckman and Payne, 1981).

Tessier *et al.*, (1990) reported that peas plant resistant to *Fusarium oxysporum* f sp. *pisi*, produced vascular responses to infection which included vessel occlusion by gel, deposition of callose in some xylem parenchyma cells and extensive vascular browning. In susceptible interactions the pathogen grew laterally from initially infected cells into adjacent cells and parenchyma cells until the vascular bundle was completely colonized. While in the resistant interactions the pathogen was confined to initially infected cells. Schroder and Walker (1942) noted increased cambial activity in infected pea plants by *Fusarium oxysporum* fsp. *pisi*.

Cells under attack by powdery mildew fungi frequently produce a papilla on the host cell wall at the site of attack, probably before the host wall is penetrated (Edwards and Allen, 1970). Host cytoplasm consistently aggregates at the site of barley attack by *E. graminis* before a papilla or a haustorium becomes visible within the host cell (William and Susan, 1974).

### 2.2.10 Control of *Cercospora* Leafspot

Few investigations have been carried out in an attempt to control *Mycovellosiella* leafspot of pigeonpea. However, various control measures have been recommended for control of other *Cercospora* leafspots. A wide range of cultural practices is commonly used in disease control. Crop losses have been significanly reduced by burning and deep ploughing infected plant debris (Hemingway, 1954). A two year crop rotation has also proved effective (Hemingway, 1954, Smith and Littrell, 1980). On maize, the disease is associated with continuous production under minimum tillage. To reduce crop loss, deep ploughing of plant residues before planting has been recommended while the advantages of minimum tillage under severe infection by *Cercospora zeae-maydis* were being re-evaluated (Hilty *et al.*, 1979). A few *Cercospora* species are seedborne (Hemingway, 1954). Use of clean certified

seeds especially in areas where the disease has not been reported is a potential exclusion measure.

Application of fungicides is a common practice, which has given encouraging results. Harrison (1969) reported 100% increase in yield and 90% reduction in defoliation when Daconil 2787 80wp was applied at 1.5 pounds per acre and Benlate (Methyl-1-(butylcarbamoyl) benzimidazol-2- carbamate) at 0.3 pounds per acre. Both fungicides were sprayed at two-week intervals. In an attempt to control groundnut leafspot, Smith and Littrell, (1980) used Benlate, copper hydroxide, chlorothalonil and mancozeb, a zinc ion and manganese ethylene bis dithiocarbamate. They realized 35.4% reduction in defoliation and 60% increase in yield. In 1988, Njeru reported low disease incidence when *Cercospora* leafspot of *Molucella laevis* was controlled using Benlate and Mancozeb. Use of resistant varieties has been singled out as the most economical measure in the control of *Cercospora* leafspots. In maize, cassava, groundnut and groundnuts, sources of resistance have been identified and resistant cultivars developed (Hemingway, 1957; Nevill, 1981).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Characterization of *Mycovellosiella cajani* the causal agent of *Mycovellosiella* leafspot in pigeonpea

### 3.1.1 Sources of pigeonpea tissue infected by *M.cajani*

Leaves, pods and petioles showing characteristic symptoms of *Mycovellosiella* leafspot of pigeonpea were collected from a pigeonpea breeding nursery of advanced lines at Kabete campus of the University of Nairobi.Other infected samples were collected from pigeonpea landlaces in Taita Taveta, Nyambene, Embu, Kilifi, Katumani and Malindi. Samples from Mbeere included those from cv. NPP 670. The infected materials were brought to the plant pathology laboratory at Kabete campus, University of Nairobi and preserved as dried specimens.

### 3.1.2 Isolation of *Mycovellosiella cajani*

Isolations were made from lesions showing fungal sporulation. In case of non sporulating lesions, the fungus was induced to sporulate by incubating the infected tissue in a moist chamber after first surface sterilizing with 10% w/v sodium hypochlorite for 5 minutes and rinsing in five changes of sterile distilled water. The mist chambers were lined with filter papers and sterile distilled water was used to keep the paper moist.

(i) **Preparation of pigeonpea leaf decoction agar plates and water agar plates.**

Pigeonpea leaf decoction agar (PLDA) was prepared using the protocol described by Njoya, (1991). Three hundred of freshly picked *Cajanus cajan* leaves were weighed and crushed in a blender with 20ml water. The mixture was filtered through a double layer of cheesecloth in a flask, sterile distilled water was added up to 1L mark to which 20g of agar was added and

gently dissolved using a heated magnetic stirrer. The suspension was autoclaving at 121°C for 15 minutes. Approximately 20 ml of the solution was dispensed into each of the sterile petri dishes after cooling to about 45-48°C in a water bath.

Water agar plates were prepared by adding 20 g of agar to 1 liter of water and autoclaving at 121°C for 15 minutes. Twenty ml of the solution was dispensed into each of the sterile petri dishes after cooling to about 45- 48°C in a water bath.

(ii) **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 were placed at the centre of the slide. Infected material showing fungal sporulation were placed on the stage of a binocular dissecting microscope and conidia were touched with a tip of a fine moistened mounted needle without touching the host material. The conidia were transferred to the water droplet on the slide and stirred with a wire loop. After stirring, the drop was streaked across the surface of water agar plates. Four strokes were made to distribute the spores. The plates were then incubated at 20 - 24°C. Conidial germination was monitored after every 3 hours.

(iii) **Single spore isolation of *Mycovellosiella cajani***

Pigeonpea leaf decoction agar was used to culture *M. cajani*. Water agar plates inoculated with *M. cajani* were incubated for 2 days. A single germinating conidium was cut out and transferred to pigeonpea leaf decoction agar plates, which were incubated at 20 - 24°C for 14 days.

(iv) **Maintenance of the inoculum**

Conidial suspension was prepared from plates that had been inoculated with single spores and incubated at room temperature for 14 days. This suspension was stored in vials at 4- 6°c. The fungus was subculture every 3 to 4 weeks and plates incubated for 10 - 14 days. Densely sporulating areas of the colonies were selected and used to prepare fresh conidial suspensions.

3.1.3 **Pathogenicicity test**

This investigation was conducted to provide proof that the isolated fungus is indeed pathogenic to *Cajanus cajan* and is capable of inducing the originally observed symptoms of *Mycovellosiella* leafspts.

(i) **Test plants.**

Pathogenicity test was conducted using NPP 670, which is an advanced breeding line of pigeonpea susceptible to *Mycovellosiella* leaf spot. Seedborne inocula were eliminated prior to planting through treatment with 50 %WP benomyl (trade name, benlate) at a recommended rate by DU PONT company of 20g/ 20L water. Seeds were sown in sterilized forest soil mixed with animal (cowdung) manure and stone gravel mixed in ratio of 10:1:1 (by volume) as described by Nyabundi, (1980).

(ii) **Inoculum preparation**

Conidial suspension was prepared by flooding 14 day old cultures of the pathogen (*M. cajani)* grown in pigeonpea leaf decoction agar medium with sterile distilled water. Conidia were scrapped off using the edge of a sterile glass slide and the suspension obtained was filtered through a double layer of cheesecloth and the concentration was determined using

neubauer improved haemocytometer. Thereafter, the conidial suspension was adjusted to 2.0 x $10^6$ conidia $ml^{-1}$ using sterile distilled water.

(iii) **Inoculation of test plants**

Inoculation of test plants was done 15 and 120 days after germination. Plants were inoculated using a modified inoculation technique of Van der Vossen *et al* (1976). To test whether all the isolates of *M.cajani* collected from different sites are pathogenic to pigeonpea, test plants were inoculated with different individual isolates. Conidial suspension with a concentration of 2.0 x $10^6$ $ml^{-1}$ was applied on both sides of all the leaves present on the plant using a half liter Baygon atomiser (Bayer East Africa Ltd) held at a distance of 10-15cm away until runoff was obtained. Inoculation was repeated after 48 hours interval was applied. Control plants were sprayed with sterile distilled water. To maintain high humidity, 15- day old inoculated plants were covered with transparent plastic bags for 96 hours. To increase leaf wetness plants were sprayed with water at least twice a day. The plants were incubated for 35 days in the glasshouse at an average temperature of 20-26°C.

(iv) **Re-isolation and subculturing**

Inoculated plants were examined daily for symptom development and colour, shape and size of lesions produced on the leaves were recorded. At termination of experiment (35 days after inoculation), leaves showing characteristic *Mycovellosiella* leaf spot symptoms were detached. Re-isolation of causal fungus was performed to fulfil Koch's postulates with respect to all the isolates collected from different sites.

### 3.1.4. **Reaction of pigeonpea to *Mycovellosiella cajani***

Fifty pigeonpea accessions/ lines were assessed for there reaction to *M. cajani* and these includes, MKS KB 94/1, MKS KO 161/1, KBK O/C 672/4, MKS TK 115, KBKOC 673X725, MKS TK 14, NYENDE JXP KZ, KBK O/C 672/1, KBZ CR 691/4, ICEAP 00350, MKS KO 252, NPP670, KBK T21, MKS TK 19, KO 91, KB 48, KBK OC KBZ OT (2), KBK O/C KBZ, KBT 671/1, ICEAP 00781, MARIKEBUNI, KAT 777(I-E), KBK OC (2) XQPL-3 (F3), MKS 83/1, KZ 48/2, TKA KO 199, KBK KZ 83/3, MKS KO 20, MKN KO 74, MKS TK 72, KO 16, MKS KB 106, ICP 6927(I-E), ICPL 93064, ICEAP 00535, NYENDE IXKZ 212, KO 25/2, KB 38/1, ICEAP 00336, MKS KO 31, ICEAP 00753, KBZ CR O/C 691/4, ICPL 87091, KO 31, ICPL 93015, KZ 56, MKS KO 94/2, ICPL 93020, ICEAP 00369, ICPL 86091. These were advanced breeding lines except Marikebuni, which is a landlace from coast province of Kenya.

Table 1: *Mycovellosiella* leafspot assessment scale (1- 9) grade

| Code | Description | Category |
|---|---|---|
| 1. | No visible disease symptoms | Very resistant |
| 2. | Two to three spots leaf$^{-1}$ 10% of leaf area Affected, no chlorosis | Resistant |
| 3. | Spots covering 10-20% leaflet area, no chlorosis | Moderately resistant |
| 4. | Spots covering 20-30% leaflet area, no chlorosis | Low resistance |
| 5. | Spots covering 30-40% leaflet area no chlorosis | Low resistance |
| 6. | Spots covering 40-50% leaflet area, the mild chlorotic patches on leaflet | Low susceptible |
| 7. | Spots covering 50-60% leaflet area, the entire leaflet mildly chlorotic | Moderately susceptible |
| 8. | Spots covering 60-70% leaflet area, entire leaflet severely chlorotic | Susceptible |
| 9. | Spots covering more than 70% leaflet area, severe chlorosis of entire leaflet and defoliation of one, two or three leaflets. | Very susceptible |

In broad terms grades 1,2,3 would be considered as resistant; 4, 5, 6 as intermediate and 7, 8, 9 as susceptible.

SOURCE: International pigeonpea newsletter 13 by Songa, 1991

3.1.4.1 **Greenhouse experiments**

(i) **Planting**

Pigeonpea seeds were surface sterilized using 10% sodium hypochlorite and rinsed using 5 changes of sterile distilled water. Pigeonpea genotypes were sown in paper sleeves containing a mixture of forest soil, cow manure and gravel in the ratio of 10:1:1 respectively. The bags were placed in a glasshouse and potting mixture was kept moist using tap water. A complete randomized design with 3 replicates was used.

(ii) **Inoculum preparation**

Plates with 14 day old cultures of *M. cajani* isolates collected from all the sites 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 and all the isolates were bulked together. The conidial concentration was determined by use of neubauer improved haemocytometer and adjusted to $2 \times 10^6$ conidia $ml^{-1}$

(iii) **Plant inoculation and incubation**

Inoculation with *M. cajani* at a concentration of $2 \times 10^6$ conidia $ml^{-1}$ was done using a half litre Baygon atomizer held at a distance of 10-15 cm away until runoff. Inoculation was repeated after 48 hours. High relative humidity was maintained for the first four days after the first inoculation by covering the plants with polythene sheet. To increase leaf wetness plants were sprayed with water at least twice a day. The plants were incubated for a total of 35 days on the floor of the glasshouse with average daily temperature ranged from 20 to 26°C. Daily observations on symptom development were made until the first appearance of symptoms and thereafter up to leaf defoliation.

(iv) ***Mycovellosiella* leafspot assessment**

At the seedling stage, *Mycovellosiella* leafspot assessment was done at 17, 19, 21, 23, 25 and 27 days after the first inoculation and thereafter on a weekly interval up to 4 weeks after inoculation during flowering stage. Disease scoring key adopted from Songa (1991) was used to categorize various infection levels as illustrated in table 1.

3.1.4.2. **Field experiments**

Fields studies were conducted to evaluate the relative resistance/ susceptibility of pigeonpea accessions to *Mycovellosiella* leafspot of pigeonpea. The accessions were planted at Kabete and Katumani in three replications in a completely randomized block design. Ecological conditions at each location were noted (Appendix 11 and 12, respectively).

Pigeonpea cv. NPP 670 that is highly susceptible to *M. cajani* was used as a spreader material. The spreader material was planted in rows spaced 2.25m apart in each plot. The spreader material was also used as guard rows between replications. Two 3m rows of each of the pigeonpea genotypes evaluated was planted at a spacing of 30cm within rows and 75cm between rows. The rows were planted at right angle to and in between the pairs of parallel spreader rows. A space of 1m at each end of the test rows separated them from the spreader rows. The replicate blocks were bordered and separated by a pair of spreader rows.

Weeding was done by hand wherever necessary. Regulars scouting for insect pests were conducted and appropriate control measure taken. The common pests noticed were caterpillars (borers), leaf and flower chewing beetles and aphids. A regular spray of Thionex

(endosulfan) controlled the insect pests at 50ml per 20 litres of water at 7 days intervals untill the pods matured. A total of 3 sprays were applied.

Artificial inoculation with bulked isolates of *M. cajani* at a concentration of $2 \times 10^6$ conidia per ml was done when most of the early maturing genotypes were at flowering and podding stage. Leaves were inoculated by spraying on both surfaces using a half litre Baygon atomiser held at a distance of 10-15 cm away until runoff. A double inoculation at 48 hours interval was applied.

3.1.4.2(i) ***Mycovellosiella* leafspot assessment**

Fifteen days after inoculation, *Mycovellosiella* leafspot assessment was done and then repeated on a weekly interval up to 4 weeks after inoculation, using the 1-9 score described in section 3.1.4

3.2. **Histological reactions in susceptible, moderately resistant and resistant pigeonpea Genotypes inoculated with *M. cajani***

3.2.1. **Test plants**

Histopathological studies were conducted using eight pigeonpea cultivars, three of which are susceptible (MKS KO 161/1, MKN KO 115, MKS KO 252), three resistant (KZ56, ICPL 87109, ICEAP 00554) and three intermediate resistant (NPP 670, MKN KO 74 and ICPL 93015) to *M. cajani*. Seeds were sown in steam sterilized forest soil, cow manure and some gravel mixed in ratio of 10:1:1 by volume (Nyabundi 1980).

3.2.2 **Inoculation of test plants**

Inoculation of test plants was done 120 days after planting. Plants were inoculated using a modified inoculation technique of Van der Vossen and co-workers (1976). All the isolates of

*Mycovellosiella cajani* collected from different sites were bulked together and adjusted to 2 x $10^6$-conidia $ml^{-1}$ The suspension of *M. cajani* was applied on both sides of all the leaves present on the plants using a half litre Baygon atomizer (Bayer East Africa Ltd.) held at a distance of 10-15 cm away until runoff. A double inoculation after 48-hour interval was applied.

3.2.3. **Light microscopy**

(i) **Leaf sampling and clearing**

Leaf tissue discs were cut out using a 1- cm- diameter cork borer after 6, 12, 24 and 48 hours after inoculation and thereafter on a daily basis until the 15th day. Leaf discs from each harvest were cleared by placing them in a pyrex bottle containing carnoy's solution (glacial acetic acid and absolute ethanol at a ratio of 1:2 (v/v) for 24 hours. The cleared leaf discs were mounted in lactophenol - cotton blue on clean slides for microscopic observations under a light microscope. Four discs were examined for each pigeonpea line/ accession in each harvest. Observations were made on mode of conidial germination, penetration and colonization of the host cells by *M. cajani*.

Semi-thin sections (1.0-1.5μm) of resin embedded material were prepared as for transmission electron microscope. They were mounted and stained for one minute with 1% (w/v) toluidine blue in 1% borax solution. The sections were viewed under a light microscope and photographs were taken where appropriate.

### 3.2.4 Transmission electron microscopy

**(i) Preparation of fixatives, buffers, dehydrating solution, embedding media and stains**

Preparation of pipes (Piperazine - N, $N^1$ - bis (2 ethanosulfonic acid) pH 8.0 and 6.8. This buffer is based on 0.3 m piperazine -N, $N^1$ - bis (2 ethanoesulfonic acid). Reagents are pipes and 0.1m sodium hydroxide (4.0g NaoH in 1L of water). Ninety grams of pipes was added to 50ml water while stirring until the powder dissolved. This happened between pH 5.5 and pH 6.0. NaoH was added until pH of 8.0 and 6.8 were reached. The solution was made upto 100 ml using distilled water. The buffer was diluted using distilled water to make up to 0.1M pipes. Glutaraldehyde fixation solution was prepared by mixing 2 ml of 5% Glutaraldehyde with 6 ml of 0.1 m piperazine - N,$N^1$ - bis (2 ethanosulfonic acid) buffer pH 8.0 and 12 ml water. To alter osmolarity, 0.4-g sucrose was added to the fixative. Osmium tetroxide was prepared by dissolving 1g osmium tetroxide in 25 ml-distilled water. Two hundred µm of pipes buffer 6.8 pH was mixed with osmium tetroxide in equal volumes.

Varying concentration of ethanol (50%, 70%, 80% and 90%) were prepared by diluting absolute ethanol in distilled water to the desired concentration.

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

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

ready for use. One- percent Toluidine blue in 1% Borax solution was prepared by dissolving 1g of toluidine blue in 100ml of 1% aqueous Borax solution.

(ii) **Fixation, dehydration, embedding, staining and viewing of leaf tissues**

Tissue fixation was done according to the method described by O'Connell *et al.*, (1984). Inoculated tissues were immersed in 5% (w/v) glutaraldehyde fixative buffered in 0.1 m pipes (pH 8.0) in stoppered glass vials. The tissues were left in the fixative for 4 hours at 4° C after which they were thoroughly washed three times for 30 minutes each in 2% sucrose in Osmium tetroxide buffered in 0.2-m pipes pH 6.8 on ice for 2 hours. The leaf tissues were later washed thoroughly in distilled water, three times for 30 minutes each. The leaf tissues were then block stained in 5% uranyl acetate overnight. They were then washed in distilled water 2 times for 10 minutes each.

The tissues were passed through a sequence of increasing concentrations of ethanol at 50%, 70%, 80% and 90%, each for 10 minutes and then through three changes of 100% ethanol each for seven minutes. They were then left in a mixture of propylene oxide and absolute ethanol in the ratio of 1:1 for 10 minutes and finally in 100% propylene oxide for 10 minutes. The tissues were kept for 2 hrs in a $^1/_2$ volume of propylene oxide and $^1/_2$ volume of Araldite mixture prepared as described in section 3.2.4 and then transferred to 100% of Araldite mixture. The vials were placed on a Taab rotator overnight to facilitate infiltration of the embedding medium. The tissues were embedded in 100% Araldite mixture in agar block moulds, labeling of tissue was done and the embedding medium was polymerized by heating the moulds in a memmort oven at 60°C overnight.

The blocks with the embedded tissues were trimmed with a Reichort MT. 60 blocks trimmer to about 0.2mm on the longest side of the trapezoid. Semi-thin sections of about 1.0 to 1.5μm 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 were identified. Those blocks were further sectioned and serial ultra thin sections were picked with 400 mesh copper grids and stained with 0.2% alkaline lead acetate for 15 minutes.

The grids were viewed under the transimission electron microscope and observations were made on penetration and colonization of the tissues by *Mycovellosiella cajani*. Photographs were taken where necessary.

3.2.5. **Scanning electron microscope (SEM)**

The fixing and dehydrating agents were prepared as in section 3.2.4. Infected pigeonpea leaves were obtained as in section 3.2.3(i) and fixed in 2.5% (w/v) glutaraldehyde in 0.1m pipes buffer (pH 8.0) for 1 hour at room temperature. They were dehydrated as in section 3.2.4(ii) and mounted on metal stubs and gold coated. The specimens were viewed under a Joel JSM - T33OA SEM and observation made on conidia germination, penetration and sporulation. Photographs were taken where appropriate.

# CHAPTER FOUR

## RESULTS

*Mycovellosiella cajani* was isolated from lesions showing sporulation of the fungus. In pigeonpea leaf decoction agar colonies of 1-3 mm were obtained 16 days after incubation. The colonies appeared grey on pigeonpea leaf decoction agar (Plate 1a) and conidia were borne at the tip of conidiophores (Plate 1b). Distinct conidia scars were observed on some of the conidia. Conidia varied considerably in size and ranged from 4.5 to 6µ by 9 to 36. The shape was also variable and included subcylindric or slightly obclavate. Some conidia had one septum while others were aseptate. Occassionally conidia having 3 or 4 septa were observed. Conidiophores appeared pale brown, smooth, septate or continous, straight or slightly flexuous, irregular, cylindrical, and frequently narrowing at the base. There were some variations in conidiophore. Some conidiophores were short while others were terminal and arose from lateral branches on the external mycelial hyphae.

### 4.2 **Pathogenicity test**

Characteristic *Mycovellosiella* leafspot symptoms were observed in all the test plants inoculated with isolates of the pathogen collected from different sites. The symptoms first appeared on the under surface of leaves as small white to brown spots 14 days after plant inoculation which later became necrotic. With continued application of water on leaves, some leafspots turned ashy grey (Plate 2a) Lesion shapes varied from circular, rectangular to irregular dictated by leaf venation. Thirty days after appearance of symptoms, leaves started turning chlorotic and there was defoliation of lower leaves in all the test plants. Most of the spots were indefinite measuring 1-3 mm wide and lesions became grey at the centre.

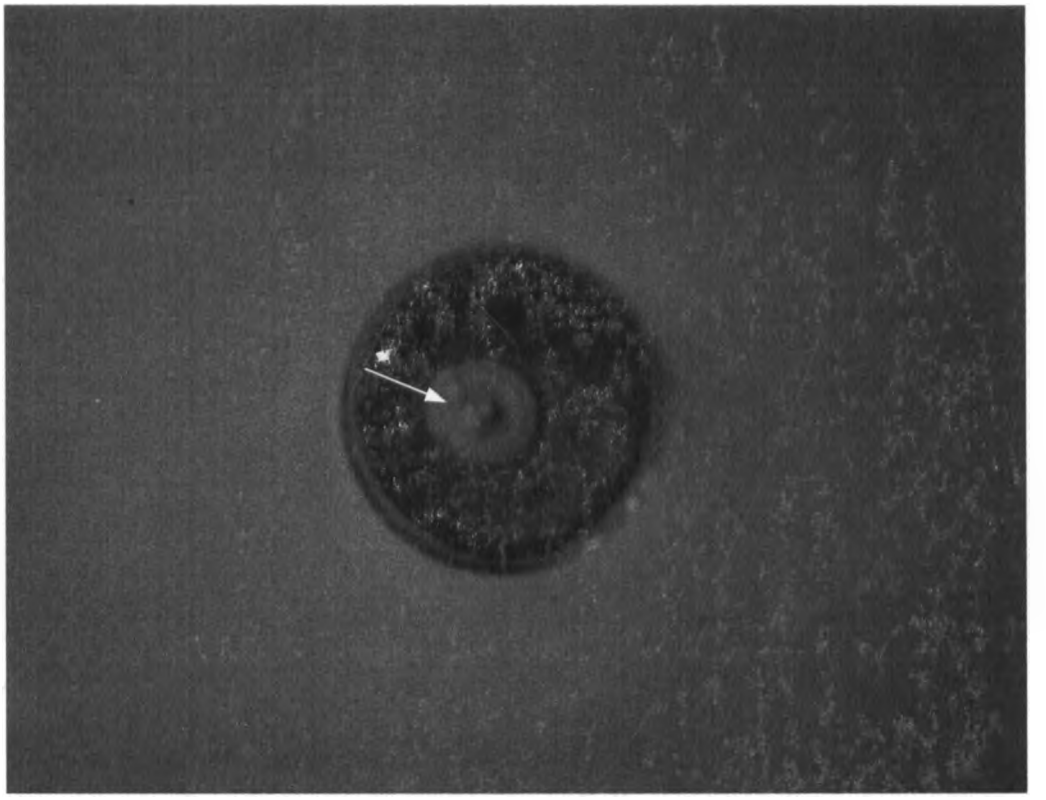


Plate 1a: A 14 day old culture (Arrow) of *Mycovellosiella cajani* resulting from a single germinating conidia used to inoculate pigeonpea leaf decoction agar media.

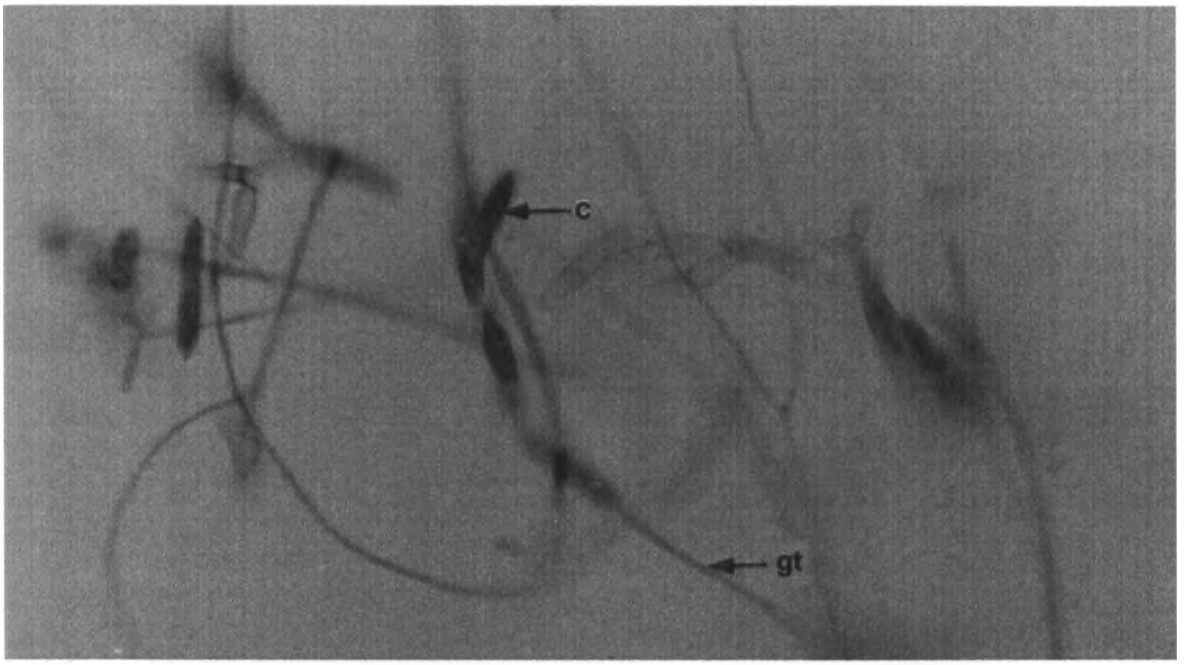



Plate 1b: Conidial germination of *Mycovellosiella cajani* at 14 days after inoculation on pigeonpea decoction agar. Magnification 384 times, c -conidia, gt- germtube

Plate 2a: Pigeonpea Cultivar NPP 670 showing leaf spots due to *Mycovellosiella* leafspot infection in the glasshouse 6 days after inoculation.
Arrow- symptoms

### 3 Screening of pigeonpea germplasm for resistance to *Mycovellosiella* leafspot

xperiments were conducted to determine the reaction of 50 pigeonpea lines to *Mycovellosiella* leafspot. The experiment was carried out both in the field and in the reenhouse. The modification of Songa, (1991) scoring method for *Mycovellosiella* leafspot iven on table 1 was used. Only infection scores were analysed statistically. In addition, ictorial diagrams (Plate 3a) given below were developed to guide the evaluator. Plate 3b is nowing *Mycovellosiella* leafspot symptoms in the field both during seedling and flowering ages.

#### 3.1 Evaluation under greenhouse condition

he greenhouse reaction of 50 pigeonpea genotypes to *Mycovellosiella* leafspot is given in ble 2.

#### i) Evaluation at seedling stage

he analysis of variance showed significance difference among the 16 medium maturity igeonpea genotypes (Appendix 1). *Mycovellosiella* leafspot severity was highest in KBK /C 672/4 (4.7) and lowest in KO 31.

he analysis of variance showed high significant (P=0.05) differences among the 34 early aturity pigeonpea genotypes (Appendix 2). *Mycovellosiella* leafspot severity was highest in IKS KO 115 (5.7). This was not significantly different (P=0.05) from ICEAP 00350 (5.2). CPL 93020, KB 48, ICP 6927 (1-E), KO 91, MKS KO 94/1, KZ 56, ICPL 93015, ICEAP 0753, ICPL 87091 and ICEAP 00781 recorded the lowest infection mean score of 1.

Thirteen pigeonpea genotypes never developed the disease symptoms at seedling stage hence were rated as highly resistant. Two pigeonpea genotypes were susceptible to *Mycovellosiella* leafspot at seedling stage, nine were low resistant, nine moderately resistant and seventeen resistant

(ii)**Evaluation at flowering stage**

In medium maturing pigeonpea genotypes, the analysis of variance showed significant (P=0.05) differences among the 16 medium maturing pigeonpea genotypes (Appendix 3). *Mycovellosiella* leafspot severity was highest in KBK KZ 83/3 (5.25) and lowest in KO 31 (1.67).

The analysis of variance for early maturing pigeonpea genotypes showed significant (P=0.05) differences among the 34 pigeonpea genotypes (appendix 4). The highest disease severity was recorded in MKS TK 115 (7.75) and lowest in KZ 56 (1.56).

In greenhouse evaluation at flowering stage, fourteen pigeonpea germplasms were rated as resistant, seventeen moderately resistant, fifteen low resistant, two low susceptible, one moderately susceptible and one susceptible.

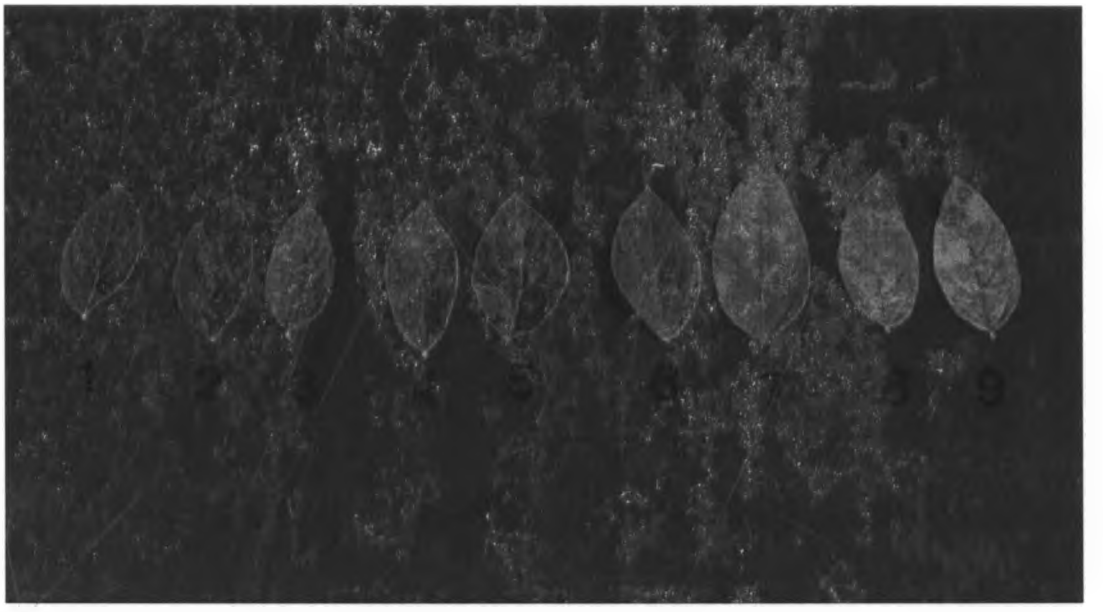


Plate 3a: Pictorial diagrams showing different levels of spotting due to infection by *M. cajani* that were used in estimating percent leaf destruction (grade 1-9 scale).

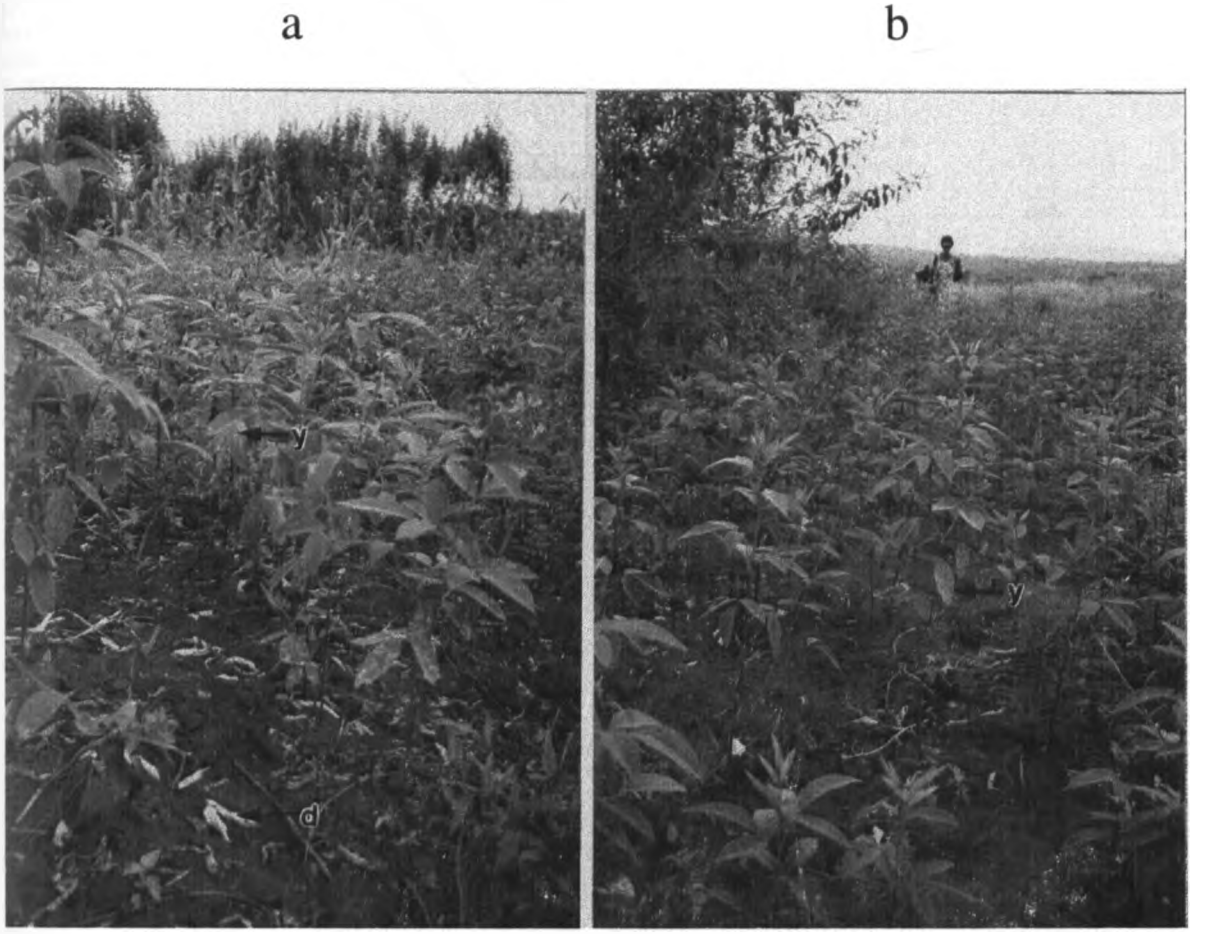



Plate 3b:Pigeonpea cultivar MKS TK 115 plants showing defoliation (d) and leaf yellowing (y) due to *Mycovellosiella* leafspot infection in the field at Katumani, both at flowering stage (a) seedling (b) stages.

Table 2 Greenhouse reaction of 50 pigeonpea cultivars to Mycovellosiella leafspot at seedling and flowering stage

| Maturity groups Genotypes | flowering infection mean score | flowering severity % | seedling infection mean score | seedling severity % | Means infection score | Means severity |
|---|---|---|---|---|---|---|
| Medium maturity | | | | | | |
| KBK KZ 83/3 | 5.25 | 38 | 2 | 9 | 3.6 | |
| KBT 671/1 | 3.9 | 22.6 | 4.4 | 30.1 | 4.2 | |
| MKN KO 74 | 3.69 | 20.6 | 2.4 | 10 | 3.0 | |
| NYENDE JXP KZ | 3.67 | 20.4 | 2 | 9 | 2.8 | |
| ICEAP 00336 | 3.57 | 19.23 | 1.2 | 0.4 | 2.4 | |
| KO 25/2 | 3.56 | 19.1 | 1.8 | 8 | 2.7 | |
| NYENDE IXKZ 212 | 3.49 | 18.6 | 1.3 | 1.6 | 2.4 | |
| KBK O/C 672/4 | 3.17 | 14 | 2.8 | 14.2 | 3.0 | |
| KBK OC 673 X725 | 3.16 | 12 | 1.8 | 8.1 | 2.5 | |
| MARIKEBUNI | 3.08 | 12 | 2.7 | 14 | 2.9 | |
| MKS KO 94/2 | 3.04 | 11.6 | 1.6 | 5.4 | 2.3 | |
| KO 16 | 3 | 10.6 | 3.5 | 20 | 3.3 | |
| MKS KO 31 | 2.63 | 8.4 | 1.2 | 0.2 | 1.9 | |
| TKA KO 199 | 2.35 | 8.4 | 3.9 | 24.6 | 3.1 | |
| KBK O/C 672/1 | 2.08 | 6 | 1 | 0 | 1.5 | |
| KO 31 | 1.67 | 2.6 | 1 | 0 | 1.3 | |
| Mean | 3.2 | 15.4 | 2.2 | 9.7 | 2.7 | |
| LSD(mean score) 0.05 | 1.65 | | 1.1 | | | |
| CV | 31% | | 59% | | | |
| Early maturity | | | | | | |
| MKS TK 115 | 7.75 | 63 | 5.7 | 41 | 6.7 | |
| MKS KB 94/1 | 6.67 | 58 | 1 | 0 | 3.8 | |
| ICEAP 00350 | 6.19 | 54.1 | 5.2 | 39 2 | 5.7 | |
| KB 38/1 | 5.83 | 48.3 | 2.7 | 14 | 4.3 | |
| MKS KO 161/1 | 5.54 | 42 | 3 | 14 5 | 4.3 | |
| MKS TK 72 | 5.36 | 40 | 1.6 | 5 | 3.5 | |
| KBK T21 | 4.88 | 34.2 | 3.6 | 20.1 | 4.2 | |
| ICPL 87091 | 4.8 | 33.6 | 1 | 0 | 2.9 | |
| MKS KO 252 | 4.66 | 32 | 3.7 | 20.6 | 4.2 | |
| NPP 670 | 4.58 | 30 | 1.9 | 8.3 | 3.2 | |
| MKS 83/1 | 4.42 | 28 | 2.7 | 14 | 3.6 | |
| MKS KO 20 | 4.35 | 26.4 | 3.9 | 26 | 4.1 | |
| MKS TK 14 | 4.17 | 24.6 | 3 | 15 | 3.6 | |
| MKS TK 19 | 4.08 | 24 | 3.7 | 21 | 3.9 | |
| ICP 6927 (1-E) | 4.08 | 23 | 1 | 0 | 2.5 | |
| KBZ CR 691/4 | 3.86 | 21.4 | 1.3 | 0.9 | 2.6 | |
| KO 91 | 3.67 | 20 | 1 | 0 | 2.3 | |
| KBK O/C/KBZ | 3.66 | 19.8 | 3.4 | 19.6 | 3.5 | |
| ICEAP 00781 | 3.6 | 19.45 | 1 | 0 | 2.3 | |
| KBK OC (2) XQPL -3 (F3 ) | 3.49 | 19 | 2.5 | 10.1 | 3.0 | |
| KBZ CR O/C 691/4 | 3.44 | 18.41 | 2.8 | 14.2 | 3.1 | |
| KBK OC KBZ OT (2) | 3.41 | 16 | 1.7 | 6.1 | 2.6 | |
| ICEAP 00369 | 3.27 | 14.8 | 1 | 0 | 2.1 | |
| MKS KB 106 | 3.04 | 11 | 1.7 | 6.1 | 2.4 | |
| ICEAP 00535 | 2.79 | 10.4 | 1.8 | 6 | 2.3 | |
| ICPL 93020 | 2 68 | 10.2 | 1 | 0 | 1.8 | |
| KB 48 | 2.46 | 9.8 | 1 | 0 | 1.7 | |
| KZ 48/2 | 2.42 | 9.6 | 1.7 | 6.2 | 2.1 | |
| KAT 777(1-E) | 2.42 | 9.58 | 3.8 | 22 | 3.1 | |
| ICPL 93015 | 2.38 | 8.61 | 1 | 0 | 1.7 | |
| ICPL 87109 | 2.35 | 8.6 | 1.2 | 0.3 | 1.8 | |
| ICEAP 00753 | 2.05 | 4 | 1 | 0 | 1.5 | |
| ICPL 930 64 | 1.69 | 3 | 1.5 | 5.2 | 1.6 | |
| KZ 56 | 1.56 | 1.4 | 1 | 0 | 1.3 | |
| Mean | 3.88 | 22.8 | 2.2 | 9.9 | 3.0 | |
| LSD (mean score) 0.05 | 2.01 | | 1.65 | | | |
| CV | 31.80% | | 10.9% | | | |

4.3.2 **Field reaction of the pigeonpea genotypes to *M. cajani***

Planting in the field was done during the short rains October 1997 both at Katumani and Kabete. Although the crop was inoculated with *Mycovellosiella cajani*, natural infection was noted and the crop was infected as early as one month after planting at Katumani. Infection mean scores and percentage severity for both Kabete and Katumani are given in table 3.

(i)**Evaluation at Kabete Field Station**.

Among the medium maturing pigeonpea genotypes the analysis of variance showed significant (P=0.05) differences among 16 medium maturing pigeonpea genotypes (Appendix 5). *Mycovellosiella* leafspot severity was highest in NYENDE JXP KZ (6.1), which was not significantly different (P=0.05) from KBK OC 673 x 725 (5.8) and KBK 672/4 (5.3). *Mycovellosiella* leafspot severity was lowest in MKS KO 31 (2.2).

Among the early maturing pigeonpea genotypes, the analysis of variance showed significant (P= 0.05) differences among 34 early maturing pigeonpea genotypes (Appendix 6). *Mycovellosiella* leafspot severity was highest in MKS KO 161/1 (7.2), which was not significantly different from MKS TK 115 (7.1), MKS KB 94/1 (7), MKS TK 19 (5.7) and MKS KO 20 (5). ICEAP 00753 had the lowest *Mycovellosiella* leafspot severity (1.8).

Overall at Kabete, 8 lines of pigeonpea were very resistant, 18 were resistant, 8 were moderately resistant, nine showed low levels of resistance and 7 were moderately susceptible.

(ii)**Field evaluation of pigeonpea germplasm at Katumani**

Among the medium maturing genotypes, The analysis of varience showed significant difference among the 16 medium maturing pigeonpea lines (Appendix 7). The highest

*Mycovellosiella* leafspot severity was in KBK O/C 672/4 (8.4) and the lowest was in MKS KO 94/2 (1.3).

For early maturing genotypes, The analysis of variance showed significant (P=0.05) differences among 34 pigeonpea genotypes (Appendix 8). MKS KB 94/1 recorded the highest severity (9), which was not significantly different from MKS KO 161/1 (8.7) and MKS KO 115 (8.1). KZ 5 6 recorded the lowest severity (1.4).

Overall at Katumani, 14 pigeonpea genotypes were rated as resistant, 12 were moderately resistant, 10 showed low levels of resistant, 11 were moderately susceptible and 4 were susceptible. None of the genotype was immune to *Mycovellosiella* leafspot. Evaluation at Katumani registered the highest infection in MKS KB 94/1 with a mean score of 9.

**4.3.4 Correlation between greenhouse (flowering stage) and field resistance to *Mycovellosiella* leafspot**

In most cases the field and greenhouse results showed similarities in the reaction of the different pigeonpea genotypes to *Mycovellosiella* leafspot. Most of the entries rated as resistant in the greenhouse were also rated as resistant field. For instance, genotypes ICEAP 00753, ICPL 93015, ICPL 87109, KZ 56, KO 31 and MKS KO 31 showed resistance to *Mycovellosiella* in the field and greenhouse conditions. A positive and significant (P=0.001) correlation between medium maturing pigeonpea genotypes at Kabete, Katumani and greenhouse at flowering stage (Appendix 9). There was also a positive and significant (P=0.001) correlation obtained between early maturing pigeonpea genotypes at Kabete, Katumani and greenhouse at flowering stage (Appendix 10). There was a non significant correlation between greenhouse seedling and greenhouse flowering, greenhouse seedling and Kabete, greenhouse seedling and Katumani.

Table 3 Field reaction of 50 pigeonpea cultivars to Mycovellosiella leafspot at two sites in Kenya

| Genotype | Kabete infection score | severity % | Katumani infection score | severity % | Means infection score | severity % |
|---|---|---|---|---|---|---|
| Medium maturity | | | | | | |
| NYENDE JXP KZ | 6.1 | 46.0 | 6.92 | 50.4 | 6.5 | 48.2 |
| KBK OC 673X725 | 5.8 | 44.0 | 7.2 | 58.1 | 6.5 | 51.1 |
| KBK O/C 672/4 | 5.3 | 39.0 | 8.4 | 65 | 6.8 | 52.0 |
| KBK O/C 672/1 | 4.8 | 33.5 | 6.8 | 50.2 | 5.8 | 41.9 |
| TKA KO 199 | 4.5 | 30.6 | 3.8 | 23.6 | 4.2 | 27.1 |
| KO 16 | 3.5 | 18.0 | 3.4 | 19 | 3.5 | 18.5 |
| MKN KO 74 | 3.3 | 15.1 | 3.5 | 19.8 | 3.4 | 17.5 |
| KBK KZ 83/3 | 3.2 | 14.0 | 3.7 | 22 | 3.4 | 18.0 |
| NYENDE 1XKZ 212 | 3.2 | 12.0 | 2.5 | 12 | 2.8 | 12.0 |
| ICEAP 00336 | 2.9 | 10.6 | 2.3 | 9.4 | 2.6 | 10.0 |
| MARIKEBUNI | 2.8 | 10.3 | 4.1 | 30 | 3.5 | 20.2 |
| MKS KO 94/2 | 2.8 | 10.4 | 1.3 | 2.1 | 2.1 | 6.3 |
| KBT 671/1 | 2.8 | 10.2 | 4.7 | 32.1 | 3.7 | 21.2 |
| KO 31 | 2.8 | 10.1 | 1.8 | 4 | 2.3 | 7.1 |
| KO 25/2 | 2.4 | 9.4 | 2.3 | 10 | 2.4 | 9.7 |
| MKS KO 31 | 2.2 | 5.4 | 2.3 | 9.2 | 2.2 | 7.3 |
| Mean | 3.6 | 19.9 | 4.05 | 26.4 | 3.9 | 23.0 |
| L.S.D (mean score) 0.05) | 1.8 | | 1.1 | | | |
| CV | 30% | | 16.70% | | | |
| Early maturity | | | | | | |
| MKS KO 161/1 | 7.2 | 58.0 | 8.7 | 70 | 7.9 | 64.0 |
| MKS TK 115 | 7.1 | 56.1 | 8.1 | 64 | 7.6 | 60.1 |
| MKS KB 94/1 | 7.0 | 52.0 | 9 | 70.2 | 8.0 | 61.1 |
| MKS KO 252 | 6.7 | 48.0 | 5.9 | 46 | 6.3 | 47.0 |
| MKS TK 19 | 5.7 | 42.0 | 5.4 | 40 | 5.5 | 41.0 |
| MKS KO 20 | 5.0 | 36.0 | 3.6 | 20 | 4.3 | 28.0 |
| NPP 670 | 4.7 | 32.6 | 5.7 | 44.2 | 5.2 | 38.4 |
| ICEAP 00350 | 4.6 | 32.0 | 6.3 | 48 | 5.4 | 40.0 |
| MKS TK 14 | 4.6 | 31.7 | 7 | 50.6 | 5.8 | 41.2 |
| KBK O/C KBZ | 4.5 | 30.8 | 5 | 34.6 | 4.8 | 32.7 |
| KBK T 21 | 4.4 | 30.1 | 5.6 | 42.1 | 5.0 | 36.1 |
| MKS 83/1 | 4.3 | 30.0 | 3.8 | 26.4 | 4.1 | 28.2 |
| ICPL 93064 | 4.2 | 28.0 | 3 | 14 | 3.6 | 21.0 |
| KAT 777 (1-E) | 3.8 | 25.1 | 4.1 | 29.1 | 4.0 | 27.1 |
| KB 48 | 3.8 | 24.0 | 5.4 | 39 | 4.6 | 31.5 |
| KBK OC(2)XQPL-3(F3) | 3.6 | 21.0 | 4 | 29 | 3.8 | 25.0 |
| KZ 48/2 | 3.5 | 17.6 | 3.8 | 24 | 3.7 | 20.8 |
| ICP 6927 (1-E) | 3.5 | 17.4 | 3.2 | 17.6 | 3.4 | 17.5 |
| MKS KB 106 | 3.4 | 17.0 | 3.3 | 18.4 | 3.4 | 17.7 |
| KBZ CR 691/4 | 3.3 | 15.6 | 6.8 | 50.1 | 5.1 | 32.9 |
| MKS TK 72 | 3.3 | 15.3 | 3.4 | 19.6 | 3.3 | 17.5 |
| ICEAP 00535 | 3.3 | 15.1 | 2.6 | 13 | 2.9 | 14.1 |
| KBK OC KBZ OT(2) | 3.3 | 15.0 | 5.2 | 36 | 4.2 | 25.5 |
| ICEAP 00781 | 3.2 | 14.8 | 4.4 | 31.9 | 3.8 | 23.4 |
| ICPL 93020 | 3.1 | 13.6 | 1.9 | 6.1 | 2.5 | 9.9 |
| KO 91 | 3.1 | 12.1 | 5.4 | 39 | 4.2 | 25.6 |
| KBZ CR O/C 691/4 | 2.8 | 11.0 | 2.1 | 8.1 | 2.4 | 9.6 |
| ICPL 87109 | 2.7 | 10.0 | 2.1 | 8 | 2.4 | 9.0 |
| ICPL 93015 | 2.4 | 9.8 | 1.5 | 4.1 | 2.0 | 7.0 |
| ICEAP 00369 | 2.4 | 9.6 | 1.9 | 6.2 | 2.2 | 7.9 |
| KZ 56 | 2.2 | 6.0 | 1.4 | 3 | 1.8 | 4.5 |
| KB 38/1 | 2.1 | 5.2 | 2.3 | 9.6 | 2.2 | 7.4 |
| ICPL 87091 | 2.0 | 5.0 | 1.8 | 5 | 1.9 | 5.0 |
| ICEAP 00753 | 1.8 | 4.3 | 2.2 | 9 | 2.0 | 6.7 |
| Mean | 3.9 | 23.3 | 4.3 | 28.1 | 4.1 | 26.0 |
| L.S.D (mean score) 0.05 | 2.3 | | 1.12 | | | |
| CV | 36% | | 16% | | | |

## 4.4 Histological reactions of the pigeonpea genotypes to *M. cajani*

### 4.4.1 Pre-penetration and penetration events of *Mycovellosiella cajani* on pigeonpea genotypes

Prepenetration and penetration activities of *M. cajani* were similar in susceptible, intermediate resistant and resistant pigeonpea tissues. Germination was noticed as early as $1^1/_2$ hours after inoculation in susceptible cultivars. (MKS KO 161/1 and MKS TK 115). Spore germination on MKS KO 161/1 (susceptible) and MKN KO 74 (intermediate resistant) occurred 3 hours after inoculation and germtubes were produced at either one or both tips of the conidia while others emerged from the sides. In MKS KO 161/1 germtube growth was rapid and side branches were produced near the stomata and grew directly into the opening (Plate 4a). On KZ 56, most of the conidia had not germinated 3 hours after inoculation (Plate 4b). In KZ 56, germtube growth was rapid and extensive but no stomatal ingress was observed instead growing of germtube passed immediately beside or across them. More penetrations occurred in MKS KO 161/1 24 hours after inoculation. Fifty days after inoculation, conidiophores were observed in susceptible and intermediate resistant varieties and were intertwined forming rope like structures (Plate 4c). In resistant varieties conidiophores were not observed.

### 4.4.2 Colonization of pigeonpea tissue by *M. cajani*

*Mycovellosiella cajani* hyphae grew rapidly in susceptible tissue of MKS KO 161/1 and MKN TK 115 and were observed in adjacent epidermal cells from the site of penetration, deep into mesophyll and near the lower epidermis of the leaf within 12 hours after inoculation (Plate 5a). Cell collapse was seen in mesophyll directly below the penetration site and in surrounding tissue of KZ 56 (resistant) 24 hours after inoculation. In resistant varieties (KZ 56 and ICEAP 00554) hyphae were limited to the epidermal cells penetrated first, or to a few adjacent cells about 18 to 24 hours after inoculation. No hyphal growth was observed

after 24 hours. Cells in close proximity to hyphae were in advanced stage of collapse while other cells in the area appeared normal (Plate 5b). Penetration activities of *Mycovellosiella cajani* were similar in resistant, intermediate resistant and susceptible tissues

*Mycovellosiella cajani* was able to penetrate the leaf epidermis and develop through most of the inner leaf tissues, including the cuticle, epidermis, parenchyma cells and vascular bundles in 48 to 72 hours post-inoculation in pigeonpea cultivars MKS KO 161/1 and MKN KO 115. Fungal invasion correlated with severe host cell damage preferentially located in the epidermis and outer parenchyma tissues in resistant varieties. The fungus penetrated the host mostly through the stomata (Plate 6a (i)) and also depression of the cuticle was evidenced (Plate 6a (ii)) hence direct penetration.

Hyphae invaded the epidermis by directly penetrating the host cell walls. During pathogen ingress in the outer leaf tissues, penetration of host cell walls by constricted hyphae was observed frequently. Penetration channels were narrower than the hyphal diameter, and fungal ingress generally was associated with slight wall displacement (Plate 6b, arrows).

Fungal invasion coincided with severe host alterations, including organelle disintegration, cytoplasmic aggregation, swelling (Plate 6c, double arrow), and shredding of host walls (arrow) in resistant genotypes. Frequent disruption occasionally lead to tissue maceration. Invading hyphae displayed a typical ultrastructure characterized by a dense cytoplasm. One of the most typical reaction features in the invaded outer parenchyma cells was the coating of some intercellular spaces with a band of electron opaque material (Plate 6d, arrows).

SEM observations revealed that primary germtubes were initiated within 3 hours after inoculation and conidial germination peaked 36 hours after inoculation in susceptible varieties. Penetration occurred through the stomata but it was inhibited in the resistant variety (KZ 56) by production of stomatal exudates (Plate 7b). Non gladular trichomes (NGT) which were hair like structures from the plant seemed to act as a barrier to stomatal penetration by the fungus both in susceptible and resistant varieties. Stomata in KZ 56 were not usually entered by hyphae, which passed immediately across or beside them (Plate 7c). Conidiophores were produced 16-20 days after inoculation and this was evidenced in susceptible cultivars like MKS TK 115 (plate 7d). Conidiophores bearing abundant conidia were observed 20 days after inoculation in susceptible varieties and conidiophores were not observed in resistant and intermediate resistant varieties even after 20 days.

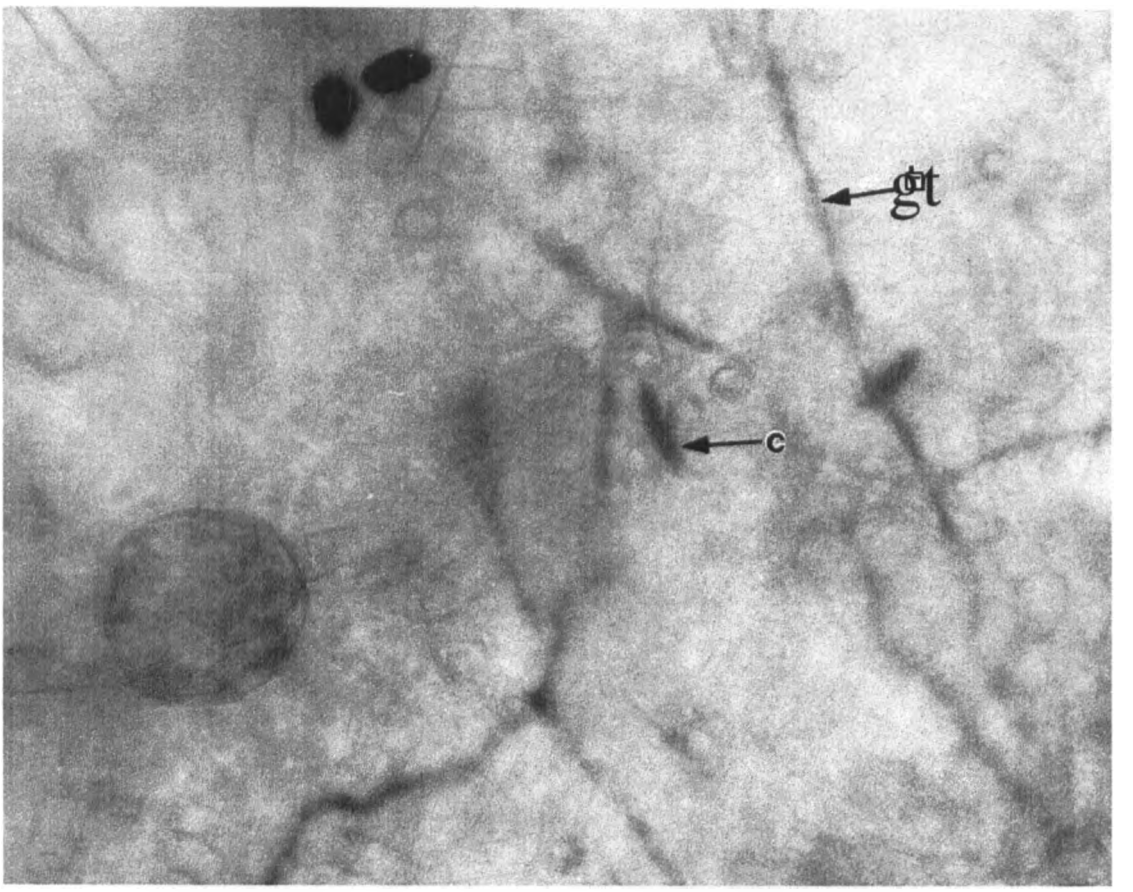



Plate 4a: Light micrograph of germinating conidia on pigeonpea susceptible (MKS KO 161/1) leaf disk after 6 hours of inoculation. Magnification- 240 times
c- conidia, gt- germtube

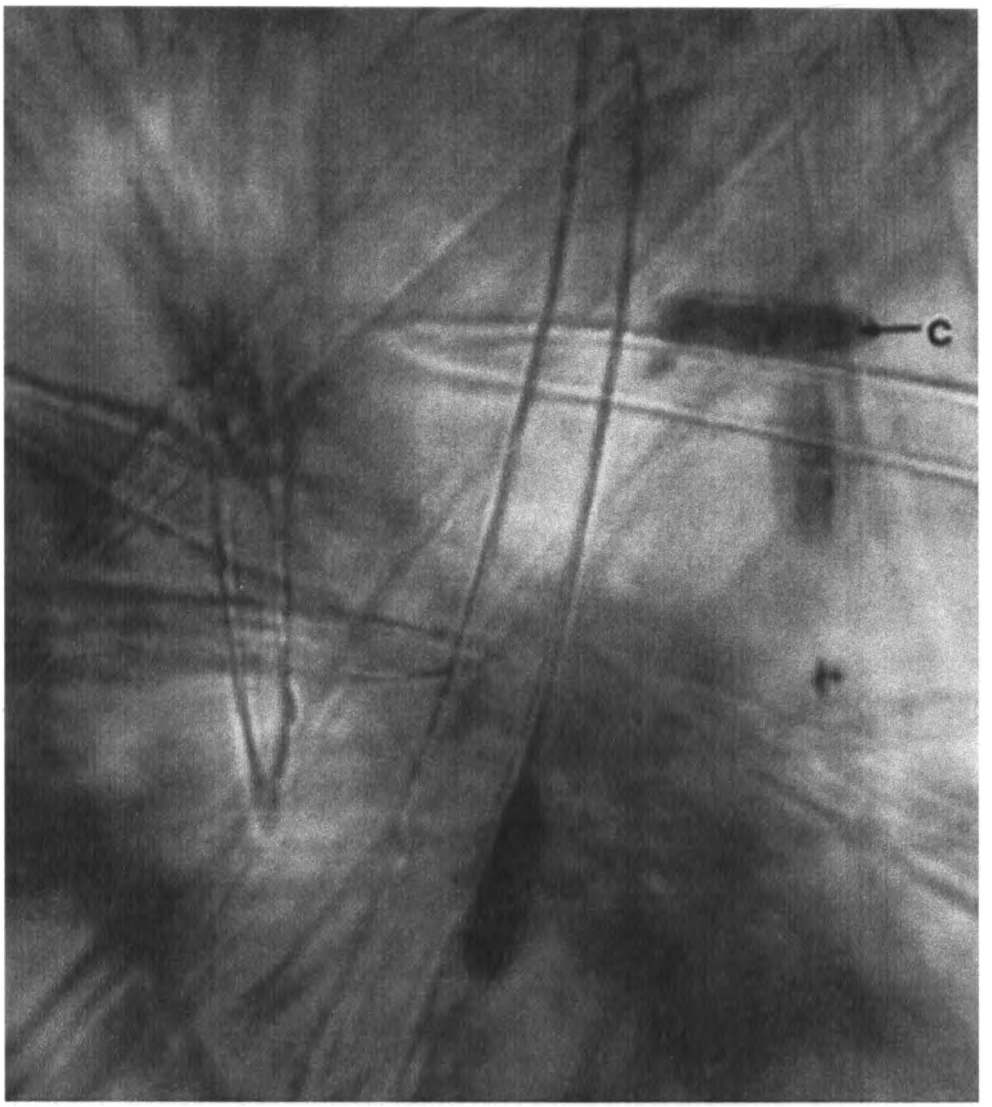



Plate 4b: Light micrograph showing no conidia germination on resistant (KZ 56) leaf disk 6 hours after inoculation. Magnification- 500 times, c- conidia

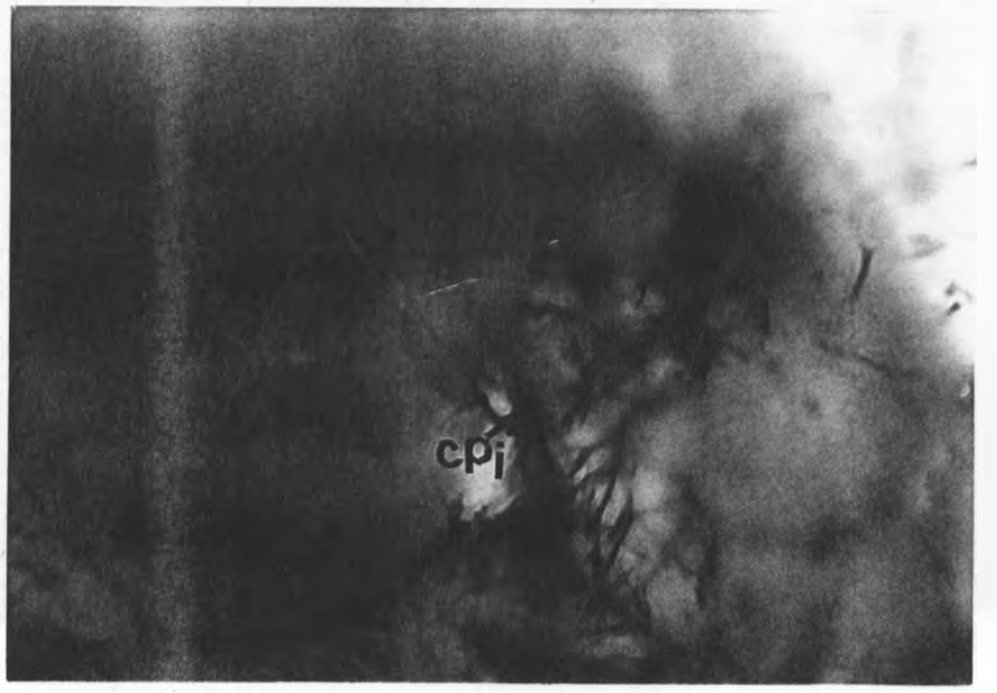



Plate 4c Spolulation of *Mycovellosiella cajani* on leaves of *cajanus cajan* showing conidiophore formation that are branched and intertwined in MKS KO 161/1 (a susceptible) variety forming rope like structures. Cpi- intertwined conidiophores

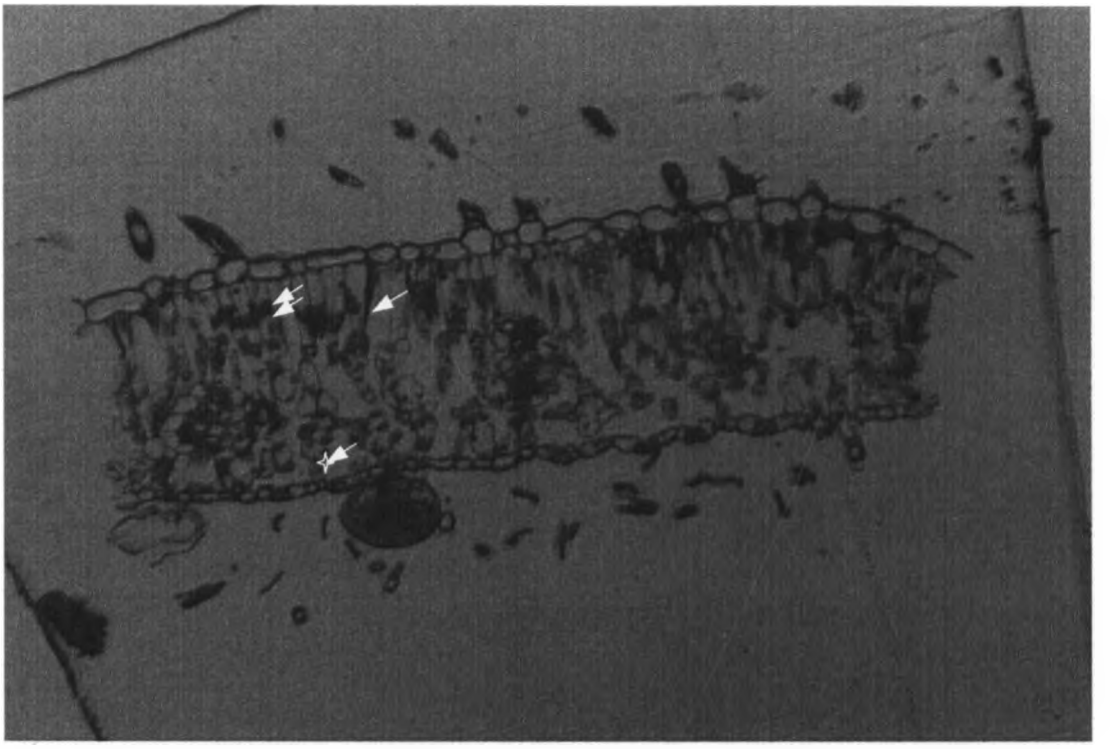


Plate 5a: Light micrographs of *Mycovellosiella cajani* in susceptible (MKS KO 161/1) pigeonpea genotype 12 hours after inoculation. Magnification 125 times, Arrow- intercellular hyphae, Double arrows- intracellular hyphae, Arrow with a star head- intact plant cell invaded by the fungus

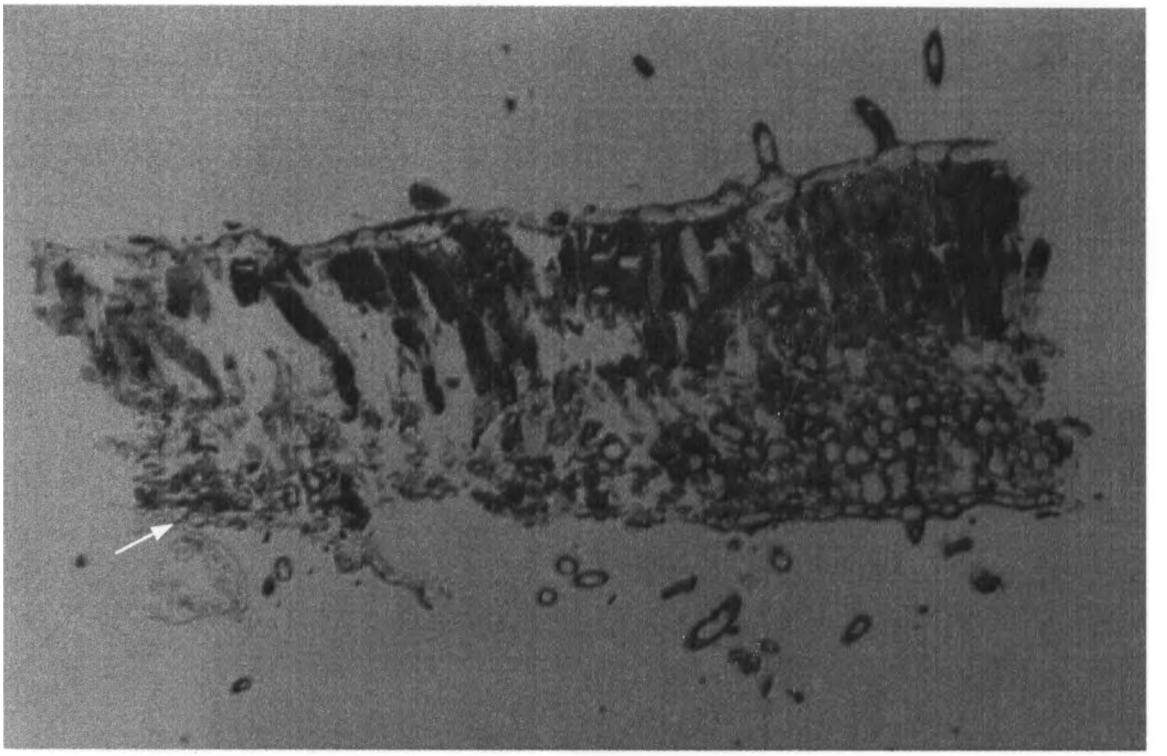
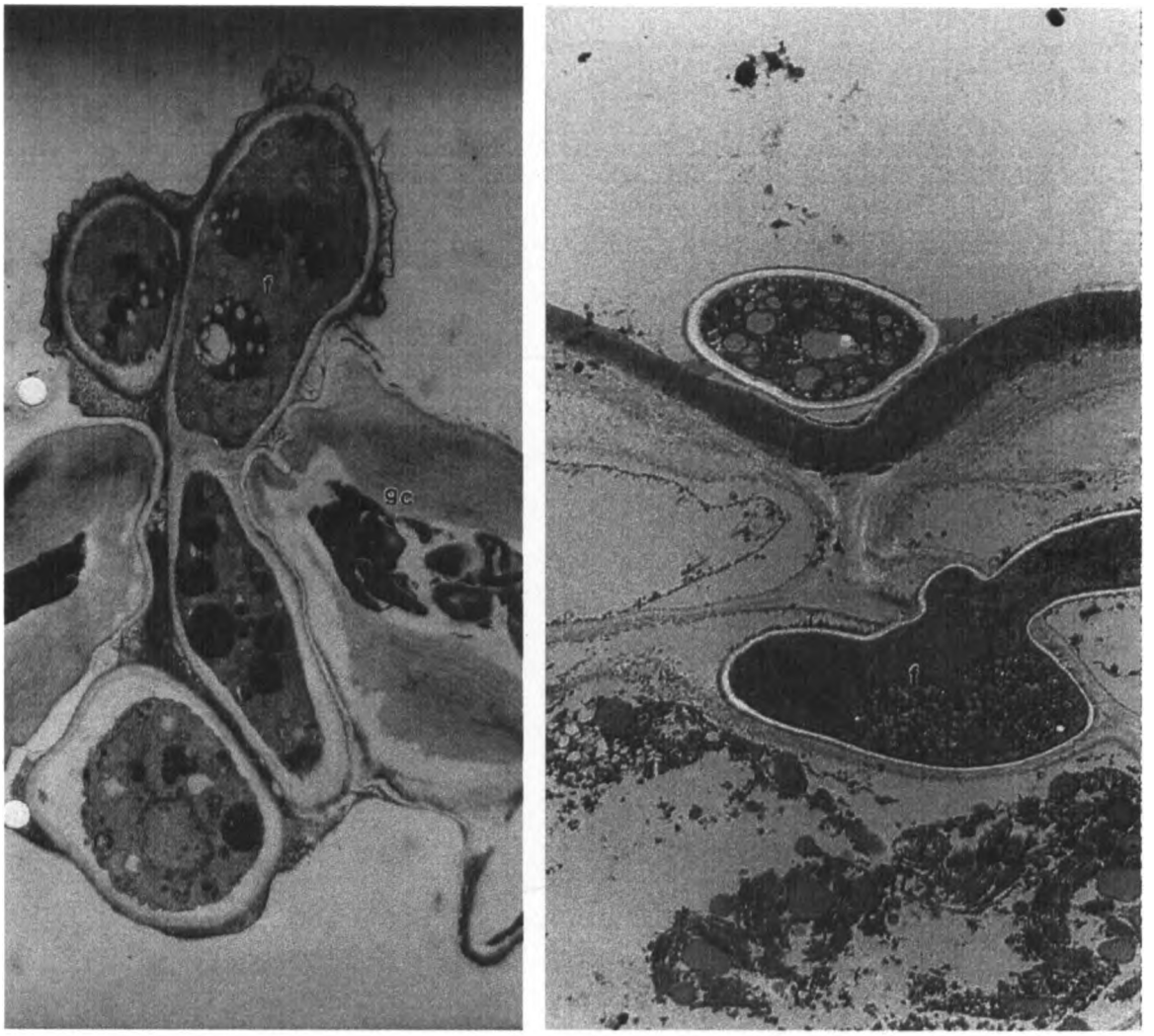


Plate 5b: Light micrograph showing cell collapse in genotype KZ 56, a resistant variety 24 hours after inoculation with *M. cajani*. Magnification– 125 times, Arrow- cell collapse




(i) (ii)

Plate6a: Transmission electron micrograph showing fungal penetration through the stomata (i) and (ii) direct penetration. Magnification- 8940 times, f-fungus , gc-guardcells

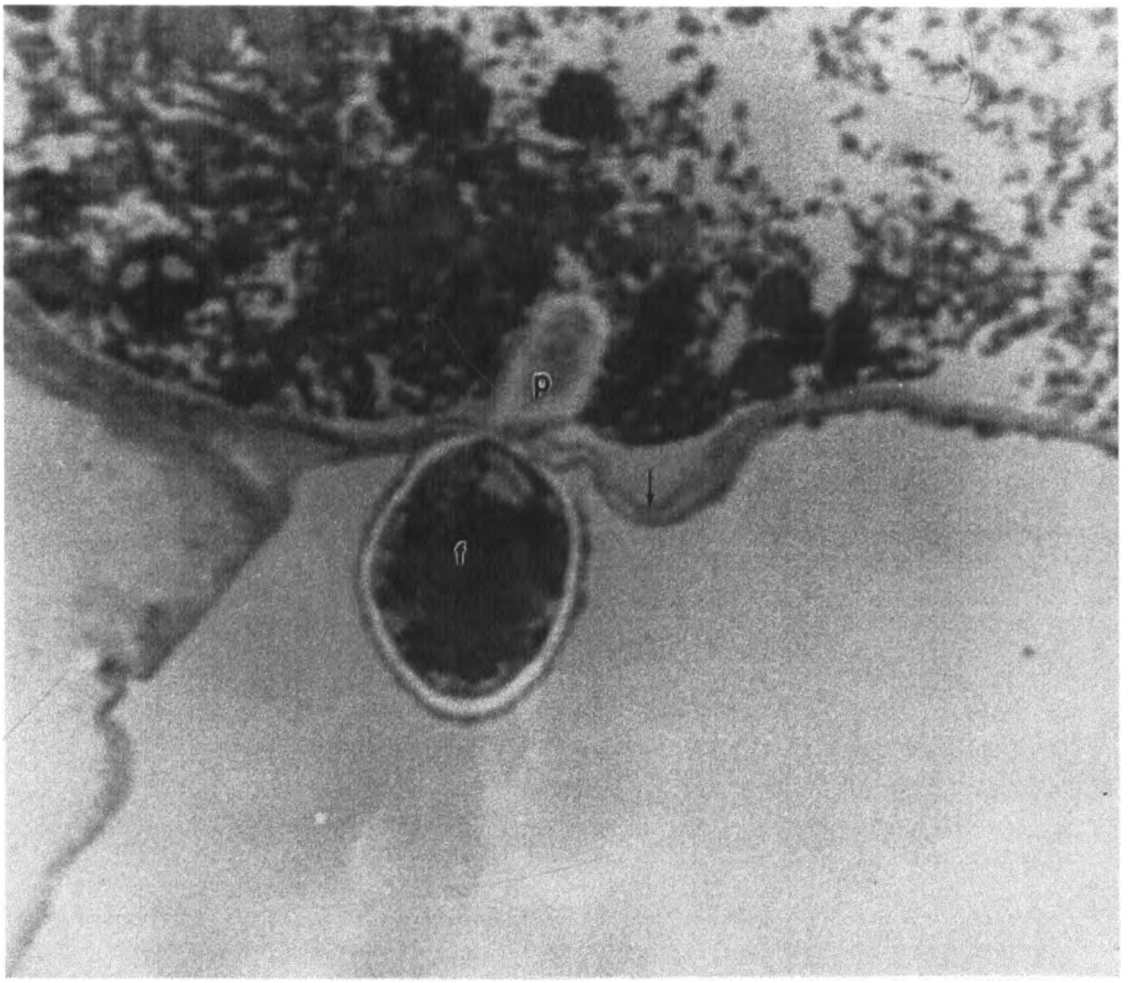



Plate 6b: Transmission electron micrograph showing slight wall displacement due to narrower penetration channels than hyphal diameter during fungal ingress. Magnification - 2820 times, f- fungus, p- penetration peg, arrow- wall displacement

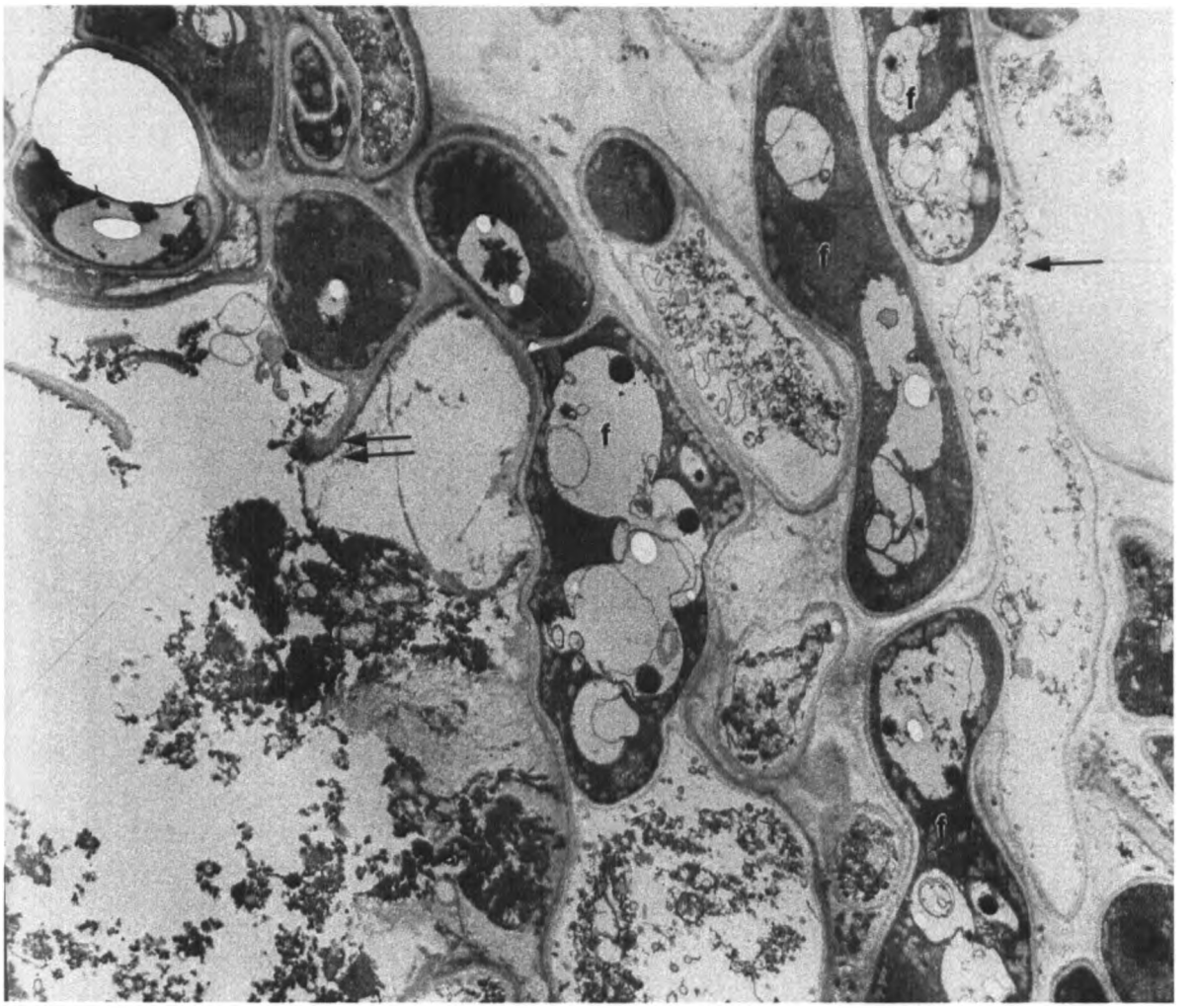



Plate 6c: Transmission electron micrographs showing organelle disintegration, cytoplasm aggregation, swelling (double arrow) and shredding of host walls ( arrow ). Magnification - 8940 times, f- fungus

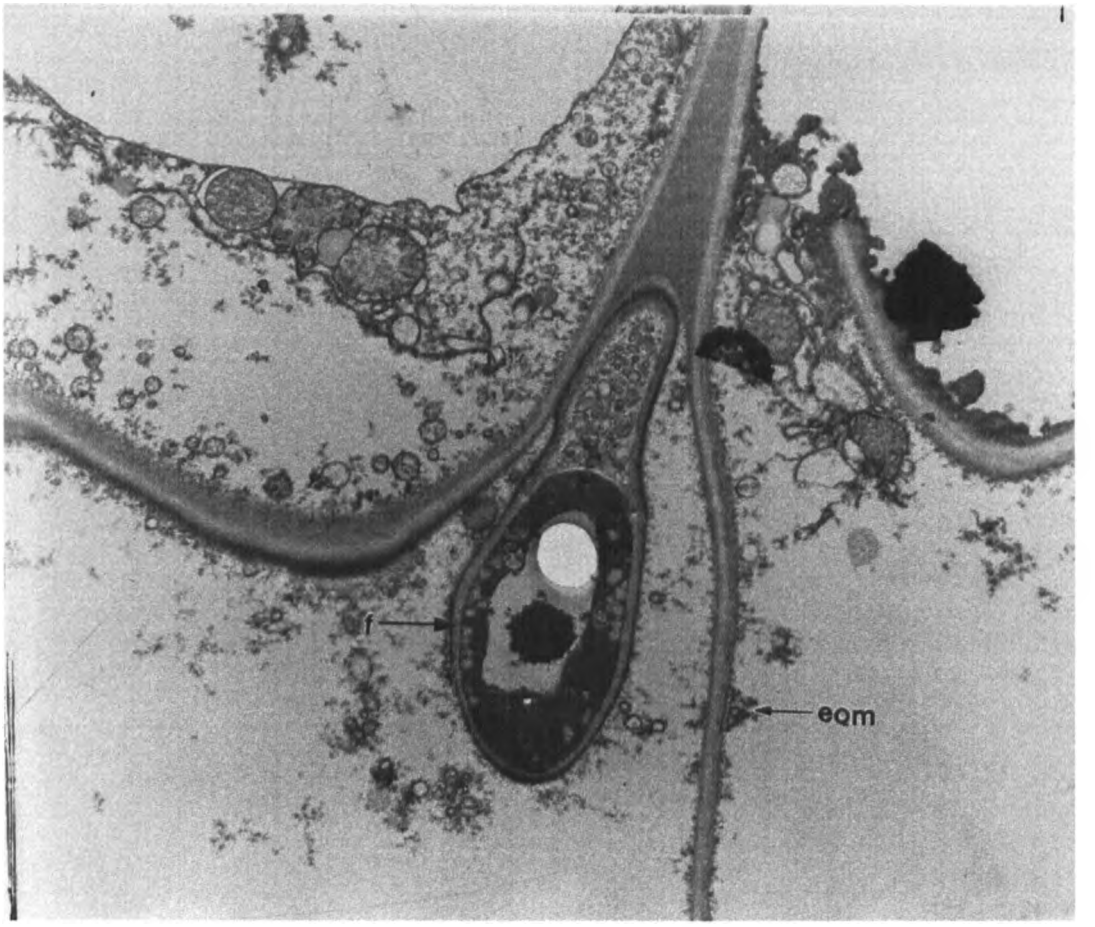



Plate 6d: Transmission electron micrograph showing intercellular spaces coated with a band of electron opaque material. Magnification-13440 times, f-fungus, eom-electron opaque material

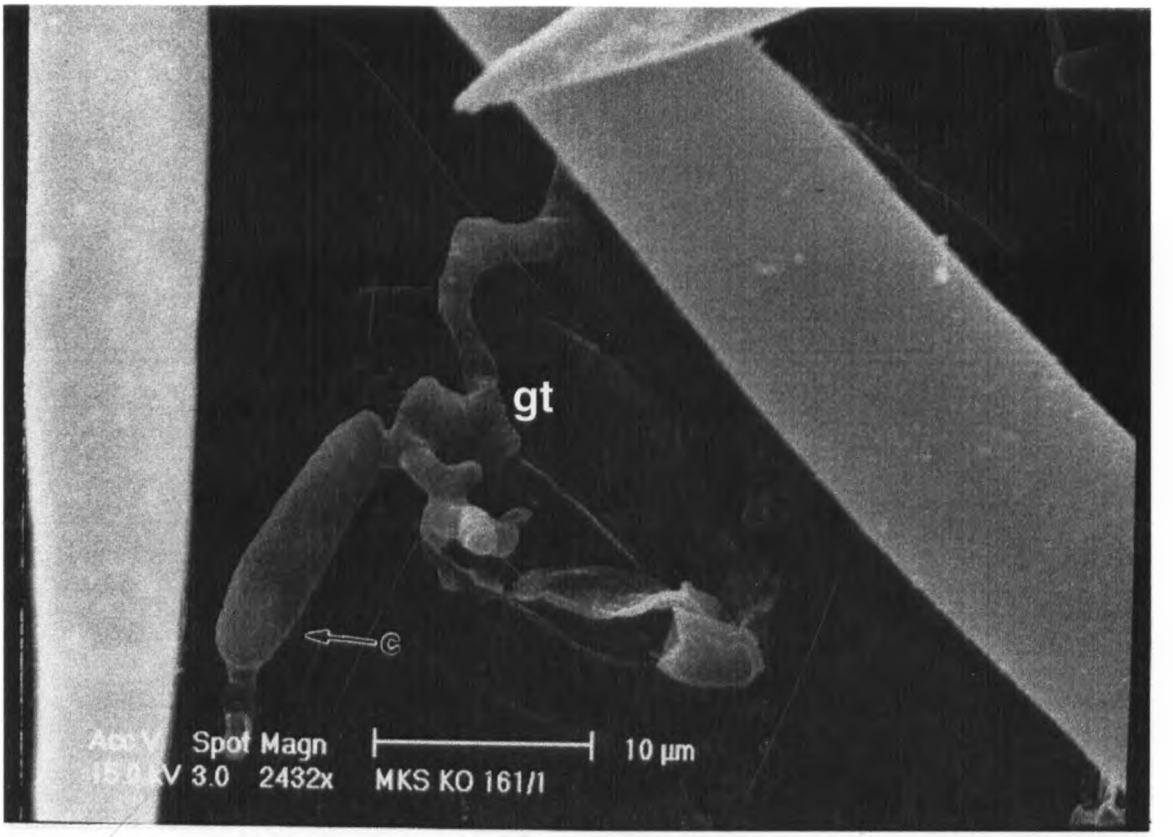



Plate 7a: Scanning electron micrograph showing conidia penetration through the stomata 6 hours after inoculation in MKS KO 161/1. c- conidia, gt- germtube

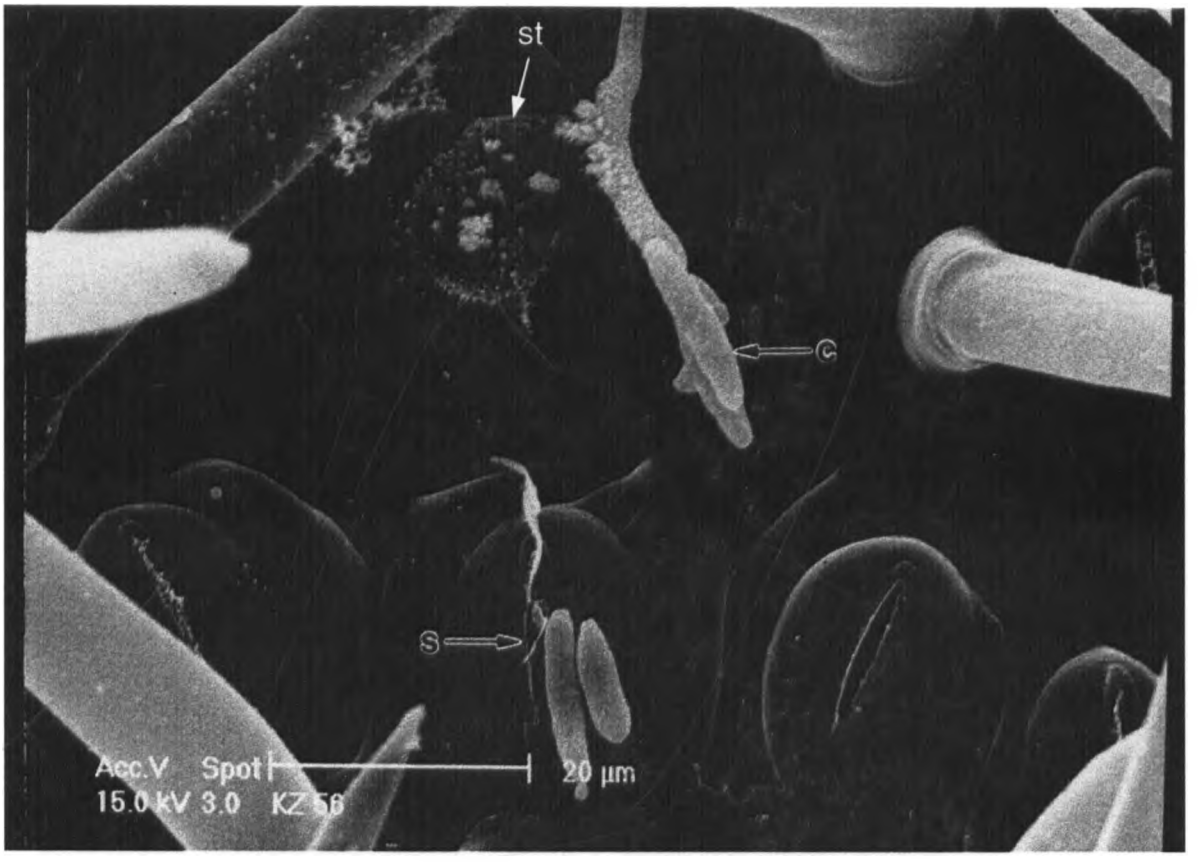



Plate 7b: Scanning electron micrograph showing penetration inhibition in KZ 56 by production of stomatal exudates. s- stomata, c- conidia, st- stomatal exudates

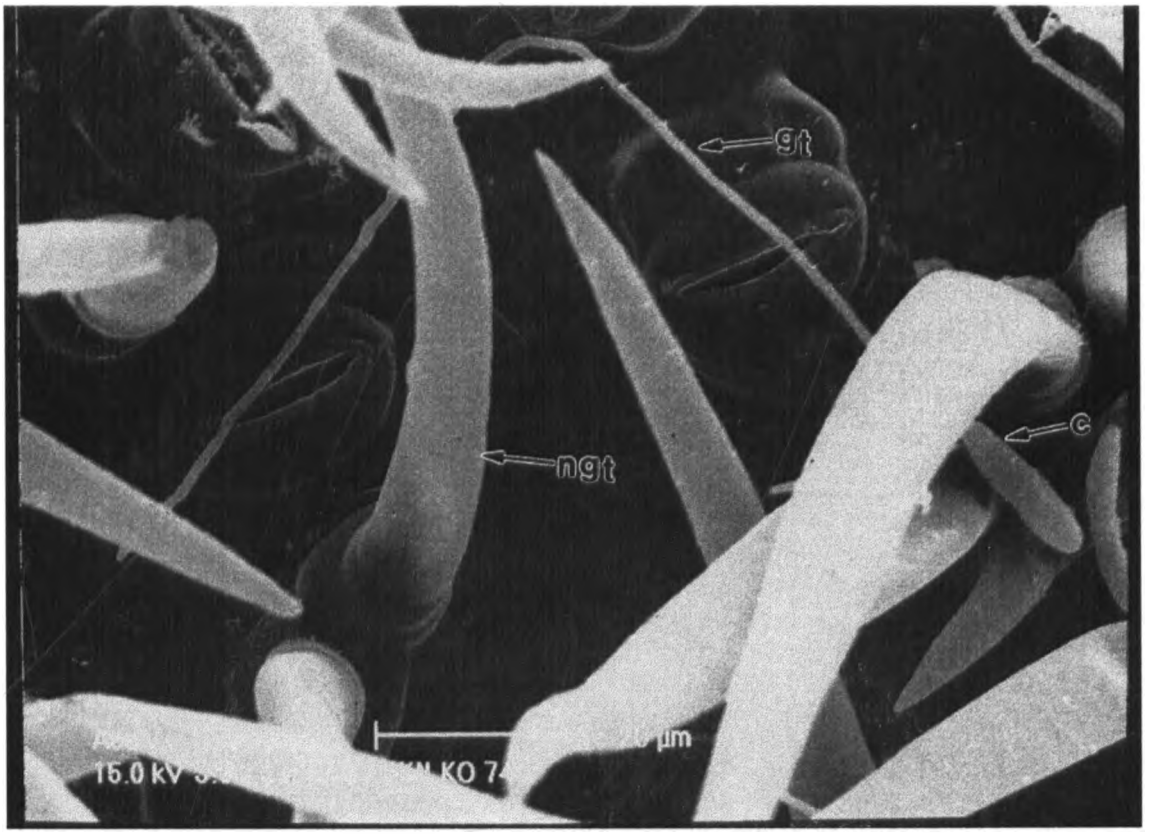



Plate 7c: Scanning electron micrograph showing germtube that passed immediately across or beside the stomata in MKN KO 74. ngt-non gradular trichome, gt -germtube, c-conidia

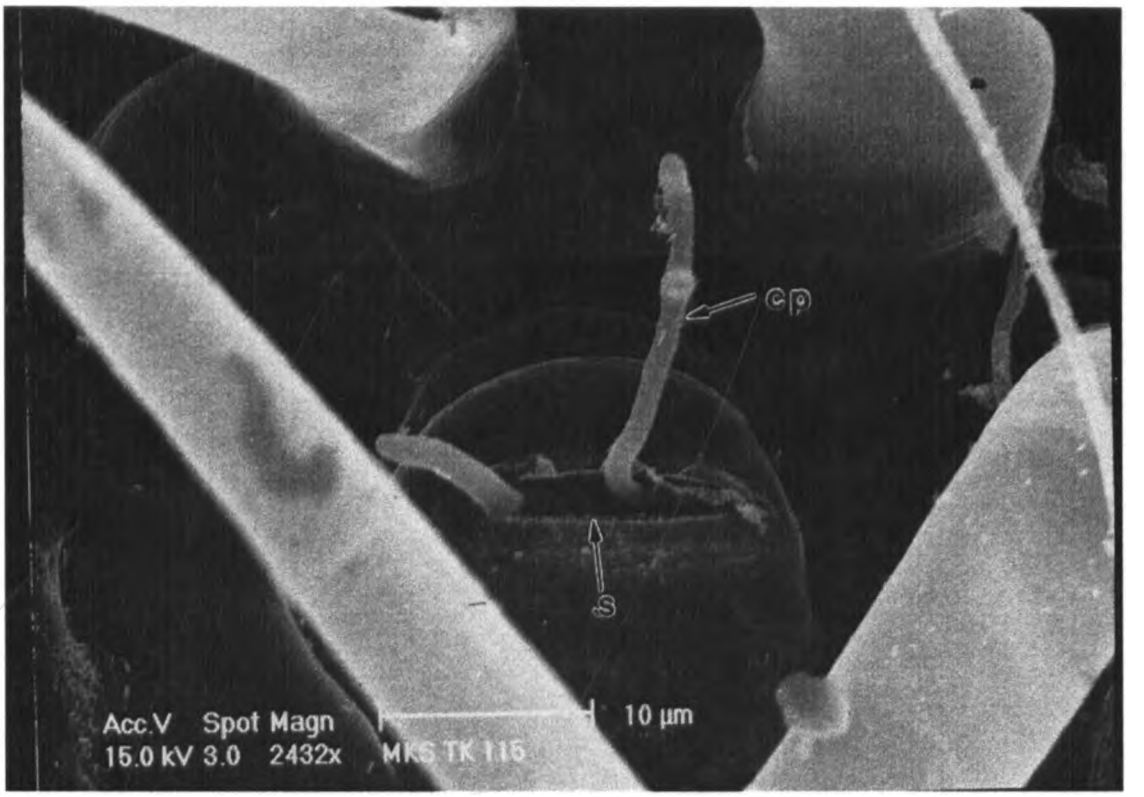



Plate 7d: Scanning electron micrograph showing conidiophore production in cv. MKS TK 115 18 days after inoculation., s- stomata, cp- conidiophore

# CHAPTER FIVE

# DISCUSSION

## 5.1 **Isolation of *Mycovellosiella cajani***

The isolated *Mycovellosiella cajani* obtained from sporulating lesions formed colonies that were grey in colour were grown on pigeonpea decoction agar. The conidia were borne at the tip of conidiophores. These observations are in agreement to those reported by Njoya (1991) on *M.cajani*. There were slight differences in dimensions and shapes of the conidia and this could be attributed to natural variations within the pathogen and environmental conditions.

## 5.2 **Pathogenicity test**

The results showed that infections first appeared on the under surface of leaves as small white brown spots and later turned necrotic. These observations are in agreement to those reported by Njoya (1991) on *M.cajani*. Lesion shapes varied from circular, rectangular to irregular as dictated by leaf venations, as frequently observed by Deighton (1974).

## 5.3. **Reaction of pigenpea genotypes to *Mycovellosiella* leafspot**

*Mycovellosiella* leafspot was found to be the most severe leaf disease that was found attacking pigeonpea plants at Kabete and Katumani (Songa, 1991). The disease was also noted in Kilifi, Malindi, Taita Taveta, Mbeere and Tharaka Nithi districts where infected samples were collected for pathogen isolation (Pers. Comm. A.W. Mwang'ombe). The disease caused yellowing and severe defoliation in pigeonpea plants in the experimental plots both at Katumani and Kabete Experimental plots were artificially inoculated. There was natural infection that was noted prior to plant inoculation.

There was a positive and significant correlation obtained between greenhouse and field results implying that both methods were reliable for screening pigeonpea germplasms for

resistance to *Mycovellosiella* leafspot. Thus pigeonpea germplasm can be screened against *Mycovellosiella cajani* at flowering stage. *Mycovellosiella* leafspot severity was higher in the field than in the greenhouse and this could be attributed to high El-Nino rains coupled with warm weather that created favourable conditions for the pathogen development in the field. *Mycovellosiella* leafspot was less severe at Kabete than at Katumani under field conditions could be probably as a result of the abundant moisture, optimum air temperature, in addition to the presence of inoculum as pigeonpea is more widely grown at Katumani than at Kabete.

Screening in the greenhouse at seedling stage resulted in more lines being rated as resistant than at the flowering stage in the greenhouse. Njoya (1991) made similar observations. According to Njoya (1991), pigeonpeas are most susceptible to *Mycovellosiella* leafspot at flowering stage and podding stage. Sme pigeonpea genotypes were also resistant at flowering stage (ICEAP 00753, ICPL 93015, ICPL 87109, KZ 56, KO 31 and MKS KO 31).

In general most of the pigeonpea genotypes tested exhibited intermediate reaction to *Mycovellosiella* leafspot infections such lines included, MKN KO 74,MKS KO 20 and KO 91.As mentioned earlier, most of the lines rated as resistant in the greenhouse were also rated as resistant under field conditions. Six were broadly resistant (ICEAP 00753, ICPL 93015, ICPL 87109, KZ 56, KO 31 and MKS KO 31). None of the pigeonpea lines was immune to *Mycovellosiella* leafspot.

### 5.4 **Histological reactions of pigeonpea inoculated with *M. cajani***

Colonization of pigeonpea tissue by *Mycovellosiella cajani* was similar to that reported for many *Cercospora* species in other plants (Beckman and Payne, 1982). Germtubes grew extensively over the leaf surface before penetrating. Wagara (1996) reported a similar type of

penetration on *Phaseoliopsis griseola* in beans. Penetration irrespective of the resistance level 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 by-passed the stomata and then penetrated directly. Dickinson (1949) suggested that entrance through stomata maybe a positive hydrotrophic response but not all plant pathogenic fungi make such a response. Two hypotheses have been proposed to explain the mechanism of direct penetration (Roberts and Boothroyd, 1984). One hypothesis indicates that the penetration peg gains ingress by mechanical pressure, while the second hypothesis suggested that, the fungus penetrate after having exerted chemical action that partially destroys the protective covering of the plant. Direct penetration of pigeonpea leaf by *Mycovellosiella cajani* appears to be through exerting mechanical pressure as indicated by the presence of inward depression of the cell wall at the point of penetration but this does not rule out the presence of some enzymatic activity. Colonization by *Mycovellosiella cajani* hyphae appeared to be restricted to the epidermal and the outer cell layer in resistant varieties. These has also been observed on tomato infected by *Pythium* group F (a minor pathogen ubiquitous in soilless cultures) and *Pythium uncinulatum* (a non-pathogenic species) (Rey *et al.*, 1998). The secondary walls of resistant varieties were more electron dense than walls of susceptible varieties. Tissue colonization by *M. cajani* was more extensive in MKS KO 161/1 a susceptible variety and MKN KO 74 an intermediate variety than in KZ56 a resistant variety. Mwang'ombe and Shankar, 1994 observed that on cv. Ruiru 11(resistant variety) wherever spoluration of *C. coffeanum* occurred, it was very sparse and no acervuli were formed.

Anatomical differences could be detected in resistant, intermediate resistant and susceptible reactions 4 days after inoculation. In the incompatible interactions the fungus was confined to the initially infected cells. In the compatible interactions thefungus grew laterally from the

initially infected cells into the adjacent cells and eventually into the vascular parenchyma cells. These results are in line with earlier reports on histopathology and ultrastructure of vascular responses in peas to *Fusarium oxysporum* f. sp *pisi* (Tessier *et al.*, 1990).

No further developments were observed in the resistant genotypes after 4 days, instead the fungus remained confined to the initially infected cells. The infected cells lost their dense cytoplasmic character and the fungus was difficult to detect in the cells after 8 days. This was also reported by Tessier *et al.*, (1990). Scanning electron microscopy revealed that some resistant varieties produced stomatal exudates that inhibited fungal penetration.

The intensity and time of sporulation was found to be an important distinguishing feature in the reactions of the different resistant categories to *Mycovellosiella cajani*. In MKS KO 161/1 (susceptible), spore production was abundant and occurred 6 days after inoculation but in MKN KO 74 (intermediate resistant) occurred 3 days later and was inhibited in KZ56 (resistant variety). The initial stagnation of fungal growth and the suppression of sporulation on the resistant and moderately resistant cultivars noted here is a feature also reported in other host-pathogen relations, for example, in coffee cultivars resistant to *C.coffeanum* Noack (Mwang'ombe and Shankar, 1994).

The restriction of colonization and sporulation in the resistant and intermediate resistant 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 maybe generally distributed and reduce the pathogen rate of colonization, are localized at particular impenetratable tissues and confine the pathogen to specific areas, or are features of

the outer layers, which restrict the fungus sporulation (Tessier *et al.*, 1990). 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 when the pathogen comes into contact with the host cells (Allen, 1959).

Some cells adjacent to infected cells in resistant plants rapidly became necrotic. The fungus did not penetrate such cells, which may correspond to a presumed hypersensitive response cells in the tomato- *Fusarium* interaction (Beckman *et al.*, 1989).

From the results of this study, it is clear that resistance in KZ 56 to *Mycovellosiella cajani* is due to limited conidial germination, delayed and reduced sporulation as observed in pigeonpea cultivar MKN KO 74

# CHAPTER SIX

## CONCLUTIONS AND RECOMMENDATIONS

This study has shown that some pigeonpea lines are more resistant than others to *M. cajan*. According to this work, pigeonpea germplasm can be evaluated for resistances to *M. cajani* in the greenhouse since the results are highly correlated to those under field conditions. This offers a good opportunity to screen large pigeonpea germplasm under greenhouse conditions without going to the field thus reducing costs. Thereafter, only promising lines should be further evaluated under field conditions.

From the results of this study some strategies for the management of *Mycovellosiella* leafspot can be recommended: -

I: Identified resistant lines should further evaluated or utlized in breeding programs.

2: Greenhouse evaluation of pigeonpea at flowering stage is still valuable as it gives results well correlated to field results and saves space.

3: Microscopy study should be carried out not only to evaluate the biological effect of the fungus to the physiological processes of the host plant but also to understand the complex interactions created between the fungus and the resistant and susceptible plant genotypes and also the events involved in infection processes which are important in disease control.

4: Race characterization/typing of *Mycovellosiella cajani* isolates is crucial to establish the extent of variability in virulence factors which is important in breeding programs

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## APPENDICES

Appendix 1: Reaction of medium maturing pigeonpea genotype to infection by *Mycovellosiella cajani* in the greenhouse at seedling stage

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Main Effects | | | | | |
| Genotype | 26.1 | 15 | 1.7 | 1.6 | .0000 *** |
| Error | 33 | 32 | 1.03 | | |
| Total | 59.1 | 47 | | | |

Appendix 2: Reaction of early maturing pigeonpea genotype to infection by *Mycovellosiella cajani* in the glasshouse at seedling stage

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Main Effects | | | | | |
| Genotype | 198 | 33 | 6 | 4.08 | .0000 *** |
| Error | 100 | 68 | 1.47 | | |
| Total | 298 | 101 | | | |

Appendix 3: Reaction of medium maturing pigeonpea genotype to infection by *Mycovellosiella cajani* in the glasshouse at flowering stage

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Main effects | | | | | |
| Genotype | 30.7 | 15 | 2.05 | 2.09 | 0.0395* |
| Error | 31.4 | 32 | 0.98 | | |
| Total | 62.1 | 47 | | | |

Appendix 4: Reaction of early maturing pigeonpea genotype to infection by *Mycovellosiella cajani* in the glasshouse at flowering stage

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Genotype | 203.9 | 33 | 6.18 | 4.05 | .0000*** |
| Error | 103.8 | 68 | 1.53 | | |
| Total | 307.7 | 101 | | | |

Appendix 5:Reaction of medium maturing pigeonpea genotype to infection by *Mycovellosiella cajani* at Kabete.

| Sources | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Blocks | 3.8 | 2 | 1.9 | 1.6 | .2264 ns |
| Genotype | 67.6 | 15 | 4.5 | 3.7 | .0012** |
| Error | 36.7 | 30 | 1.2 | | |
| Total | 108.1 | 47 | | | |

Appendix 6: Reaction of early maturing pigeonpea genotype to infection by *Mycovellosiella cajani* at Kabete.

| Sources | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Blocks | 15.7 | 2 | 7.8 | 4.1 | .0215* |
| Genotype | 205.3 | 33 | 6.2 | 3.2 | .0000*** |
| Error | 126.9 | 66 | 1.9 | | |
| Total | 347.8 | 101 | | | |

Appendix 7: Reaction of medium maturing pigeonpea genotype to infection by *Mycovellosiella cajani* at Katumani.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Blocks | 2.2 | 2 | 1.1 | 2.4 | .1042ns |
| Genotype | 210.4 | 15 | 14.0 | 30.8 | .0000*** |
| Error | 13.7 | 30 | 0.5 | | |
| Total | 226.2 | 47 | | | |

Appendix 8: Reaction of early maturing pigeonpea genotype to infection by *Mycovellosiella cajani* at Katumani.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Blocks | 0.7 | 2 | 0.3 | 0.7 | .4882 ns |
| Genotype | 442.7 | 33 | 13.4 | 28.6 | .0000*** |
| Error | 30.9 | 66 | 0.5 | | |
| Total | 474.3 | 101 | | | |

Appendix 9: Correlation coefficient between medium maturing pigeonpea genotypes evaluated in the glasshouse and field. n= 16

| Variable | Kabete vs. Katumani | Kabete vs GHF | Katumani vs GHF | Kabete vs GHS | Katumani vs GHS | GHF vs GHS |
|---|---|---|---|---|---|---|
| Correlation coefficient | 0.9814** | 0.8776** | 0.8959** | -0.076ns | -0.070ns | -0.090ns |

Key

* Significant at p = 0.01 ** Significant at p = 0.001

Appendix 10: Correlation coefficient between early maturing pigeonpea genotypes evaluated in the glasshouse and field. n= 34

| Variable | Kabete vs Katumani | Kabete vs GHF | Katumani vs GHF | Kabete vs GHS | Katumani vs GHS | GHF vs GHS |
|---|---|---|---|---|---|---|
| Correlation coefficient | 0.9792** | 0.9798** | 0.9884** | -0.060ns | -0.074ns | -0.084ns |

Key

* Significant at p = 0.01 ** Significant at p = 0.001

ns- non significant

Appendix 11: Meteorological data at Kabete from October 1997 - June 1998

| Month | Year | Temperature °c Maximum | Minimum | Rainfall (mm) |
|---|---|---|---|---|
| October | 1997 | 22.5 | 14.0 | 158.6 |
| November | 1997 | 21.4 | 14.5 | 324.6 |
| December | 1997 | 22.4 | 14.4 | 219.8 |
| January | 1998 | 22.7 | 14.8 | 327.9 |
| February | 1998 | 24.9 | 18.4 | 274.2 |
| March | 1998 | 23.6 | 18.4 | 101.3 |
| April | 1998 | 24.3 | 15.5 | 151.8 |
| May | 1998 | 22.9 | 14.7 | 327.2 |
| June | 1998 | 21.7 | 12.6 | 63.1 |

Appendix 12 : Meteorological data at Katumani from October 1997 - June 1998.

| Month | Year | Temperature °c Maximum | Minimum | Rainfall (mm) |
|---|---|---|---|---|
| October | 1997 | 34.5 | 16.5 | 43.7 |
| November | 1997 | 34.2 | 18.9 | 492 |
| December | 1997 | 35.6 | 19.5 | 395 |
| January | 1998 | 37.5 | 18.4 | 480 |
| February | 1998 | 35.75 | 18.7 | 190 |
| March | 1998 | 37 | 20.6 | 72 |
| April | 1998 | 34.25 | 18.4 | 173 |
| May | 1998 | 33.0 | 17.6 | 128 |
| June | 1998 | 31.8 | 15.0 | 6 |