CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Cowpea is a widely grown legume in tropical and subtropical Africa. It is mostly cultivated by small-scale (subsistence) farmers, usually intercropped with maize or sorghum. Cowpea seeds acts as a major source of protein (22-24%), vitamins and minerals (Coetzee 1995). As the cowpea plant develops, leaves are picked and prepared as spinach [morogo (Sotho)] and eaten with maize porridge.

1.2 Motivation of the study

The major constraints to farming of the cowpea plant are its susceptibility to a large number of pests and pathogens (Williams 1975). Fungal diseases infecting cowpea include seedling mortality caused by *Pythium* sp., anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. & Magn.) Bri. & Cav.; leaf spots caused by *Cercospora* and *Septoria* spp.; rust caused by *Uromyces appendiculatus* (Pers.) Frics. and *Pythium* stem rot cause by *Pythium aphanidermatum* (Edson.) Fitzp. (Onesirosan & Barker 1971; Bailey *et al.* 1990; Emechebe & Florini 1997) and *P. ultimum* Trow. (Adandonon & Aveling 2000). *Colletotrichum* brown blotch, caused by *Colletotrichum capsici* (Syd.) Butler & Busby (Pring *et al.* 1995), and *C. truncatum* (Schwein) Andrus & W.D. Moore, is one of the diseases causing significant losses to stems, pods and leaves of cowpeas (Latunde-Dada *et al.* 1996).

Anthracnose of cowpea caused by *Colletotrichum dematium* (Pers. ex Fr) Grove was reported for the first time in South Africa in 1997 (Smith & Aveling 1997). The infection process of *C. dematium* was described using light microscopy and scanning electron microscopy. The symptoms appear as tan brown discoloured lesions that gradually enlarge becoming dark brown to dark purple. Lesions on

the surface appear sunken with numerous black acervuli on the surface of the infected area (Smith *et al.* 1999).

The importance of *C. dematium* on cowpea in South Africa led to the undertaking of this project since the anthracnose caused by this fungus is a fairly new disease in South Africa. No work has been done on the interaction of cowpea and *C. dematium*, except for studies done in 1977 and 1984 (Lenne 1992) in India and Malaysia and the study by Smith *et al.* (1999).

1.3 Fundamental Objective

The broad objective of this study was to investigate the different epidemiological, biochemical, and plant-pathogen interactions between *Colletotrichum dematium* and cowpea.

1.4 Specific Objectives

In pursuit of the above objective, experiments were carried out in order to elucidate the following:

- Characterise *C. dematium* isolates collected from different parts of southern Africa and group them according to morphological characteristics, pathogenicity, vegetative compatibility groups and genetic relatedness.
- The necrotrophic strategy of *C. dematium* on cowpea stem
- Factors affecting the severity of the disease, i.e. the age at which the cowpea plant is susceptible and the effect of temperature and relative humidity on the onset of the disease.
- Determine if the colour of the seed coat has any effect on the resistance of cowpea to *C. dematium*.

1.5 Chapter Outline

The work of this dissertation is a series of different topics organised into chapters, each chapter taking the form of a paper submitted for publication in a

specific journal. Each chapter is written according to the specifications of the particular journal.

Chapter 2. The literature review mainly focuses on the origin, morphology, epidemiological aspects, chemical composition of cowpea seeds and food uses of the cowpea plant. The origin, morphology, and infection strategies of the anthracnose pathogens (*Colletotrichum* species) infecting cowpea, as well as some aspects of genetic relatedness are discussed. Included at the end of each section is a discussion on areas where there are gaps in knowledge.

Chapter 3. Forty-eight isolates of *C. dematium* were collected from infected cowpea fields in different parts of southern Africa. These isolates were characterised based on cultural and morphological characteristics as well as pathogenicity. Following this process, the isolates were grouped into vegetative compatibility groups using the nitrate non-utilising technique. The isolates were then characterised for genetic relatedness using random amplified microsatellites. (Submitted to *Journal of Phytopathology*).

Chapter 4. The infection strategies of *C. dematium* on cowpea were investigated. The focus was on the necrotrophic phase, type of acervuli formed, formation and release of conidia. (Submitted to *Mycological Progress*)

Chapter 5. The concentration of tannins in the seed coats and the location of other polyphenols in differently coloured seeds of some cowpea cultivars were determined. In order to investigate the relationship between the colour of the cowpea seed and resistance or susceptibility to *C. dematium*, biochemical, histochemical and microscopic techniques were used to differentiate between the cowpea cultivars. (Submitted to *Journal of Agriculture and Food Chemistry*)

Chapter 6. To be able to formulate a management programme against *C. dematium* on cowpea, a study on the factors affecting infection of cowpea

by *C. dematium* in the greenhouse was conducted. The age of the cowpea plant, the temperature of the greenhouse and the incubation period of the pathogen on the severity of the disease caused by *C. dematium* were investigated. (Published in *African Plant Protection 2002*).

Chapter 7. This chapter includes the general discussion, interpretation of results and the author's general comments concerning the experiments conducted and recommendations.

Chapter 8. Summary

1.6 Literature cited

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CHAPTER 2

LITERATURE REVIEW

2.1 Cowpea: Origin and physical characteristics

Cowpea (*Vigna unguiculata* (L.) Walp) is a member if the family Fabaceae and tribe Phaseoleae (Macheral *et al.* 1978 cited by Ng & Macheral 1985). Cowpea was known in India long before the days of Christ, and is also believed to have been known in Asia around the year 2300 BC and in Europe early enough to be known under the name *Phaseolos*, *Phaseolus* or *Phaselus* (Burkhill 1953). The probable centre for domestication has been a topic of major debate with several places mentioned as probable centres of domestication. Examples of such places include central Africa (Piper 1913), central and South Africa (Zhukovskii 1962) and West Africa (Lush & Evans 1981).

The centre of maximum diversity of cowpea is found in West Africa, in an area encompassing the savannah region of Nigeria, southern Niger, part of Burkina Faso, northern Benin, Togo, and the northwestern part of Cameroon (Ng & Macheral 1985). Padulosi *et al.* (1990) concluded that southern Africa is the centre of genetic diversity because the most primitive of the wild cowpea occurs in Namibia from the west, across Botswana, Zambia, Zimbabwe and Mozambique to the east, and the Republic of South Africa and Swaziland to the south. The former Transvaal in South Africa is depicted as the most probable centre of speciation of cowpea due to the presence of its wild varieties such as *var. rhomboidea*, *var. protracta, var tenuis* and *var. stenophylla*, all of which occur from the Transvaal to Swaziland, Cape Town, Zimbabwe, and Mozambique (Singh *et al.* 1997).

The cowpea plant is described as an annual or perennial crop, it can be erect, trailing or climbing with striate smooth or slightly hairy stems. The leaves are trifoliate and the leaflets are ovate or lanceolate with entire margins or 3-lobed at

the base. They possess an axillary inflorescence with a few to several white and mauve flowers measuring 15-22 mm long. The fruit is an erect, linear-cylindrical, smooth or slightly warty pod measuring 5-15 cm, depending on the variety. Seeds come in a wide range of colours, from white to dark red or black, often mottled, oblong or reniform (Fox & Young 1982).

Domesticated cowpeas are of two types, those from seasonally arid zones, which are short day plants and those from humid tropical regions, which are day neutral and tend to flower later than the short-day types (Quin 1997). Cowpea seeds germinate readily between 20 and 30^{0}C, but outside this range germination is particularly low, especially for the domesticated type (Quass 1995). The slow germination of wild types in high temperature may have evolved as a means of delaying germination in unfavourable conditions (Lush & Evans 1981). Cowpea is grown on a wide range of soils as long as the soil is well drained and the plant is acid tolerant to soils with pH 5.5-6.0. Most cowpea cultivars in the tropics and subtropical regions of Africa are grown in humid regions with an annual rainfall varying from 1500-2000 mm (Tindal 1983).

The most important feature of the cowpea plant is its ability to fix atmospheric nitrogen through symbiosis with nodule bacteria, *Bradyrhizobium* spp. In doing so, the crop does not deplete the natural reserves of soil nitrogen and many experimental findings suggest that levels of soil nitrogen increase following cowpea cultivation (Agboola 1978). In a study conducted by Lindsay & Gumbs (1993) in Trinidad, loam and clay soils were inoculated with a *Rhizobium* inoculum and mineral fertilisation. Loam soil inoculated with *Rhizobium* showed better cowpea growth than clay soil and this is mainly due to better drainage of loam soil. Generally, rotation of cereals with legumes is known to increase soil fertility due to the nitrogen fixing ability of the legume (Klaij & Ntare 1995). In addition to this, cowpea is drought tolerant and is able to maintain growth under dry soil conditions. This trait is explained by the deep rooting habit of some

varieties and therefore accounts for the crop's ability to grow and yield under the semi-desert conditions of Africa and parts of Brazil (Quass 1995, Quin 1997). Cowpeas contain polyphenols such as tannins, which are well known for reducing protein digestibility and quality. In addition to these, compounds containing antiphysiologic substances such as lectins and trypsin inhibitors are found. In an effort to produce insect-resistant varieties, breeders have sometimes increased the levels of trypsin inhibitors (Bressani 1985). A study in the levels of tannin and catechin on leaves of cowpea varieties, varied from 0.31 to 1.63% and from 143.6 mg/100 ml of leaf sample, respectively (Gatehouse & Bouter 1983).

2.2 Cowpea seeds: Morphological and chemical composition

Cowpea seeds vary markedly in size, shape and colour. They are 2-12 mm long, globular or kidney shaped (Fox & Young 1982). Seed shape is correlated with that of the pod. Where individual seeds are separated from adjacent ones during development, they become reniform, but as crowding within the pod increases, the seeds become globular. However, in subspecies *V. sesquipedalis*, the reniform shape is dominant or partially dominant to the shorter and rounded shape that is characteristic of the subspecies *V. unguiculata* (Fery 1980).

The seed coat structure has been studied by several authors, with different gene symbols attached to different seed coat types: *Lf* was a symbol used for the longitudinal furrowing (Harland 1919, Spillman & Sando 1930, reviewed by Fery 1980); *ls* for the loose testa and *hs* for hollow seeds (Krishnaswamy *et al.* 1945), *Th* and *Co* for thick seed coats (Ojomo 1972) and PC for perpendicular microsclereid arrangements (Rajendra *et al.* 1979). The gene for PC arrangements was later reviewed and renamed *Pc* by Fery (1980).

Cowpea seeds exhibit a wide range of colours, from white to green, buff, red, brown, or black; and are variously speckled, mottled, blotched or eyed (Fox & Young 1982, Chavon *et al.* 1989). Spillman (1911) stated that seed coat patterns

were inherited independently of their colour, but a common colour factor influenced the appearance of the colour. In the following study (Spillman 1912) it was explained that the general factor, designated C, was needed for expression, in combinations with genes R, U, Br, Br and N, and N and B conditioning red, buff, brown, black and blue seed coats, respectively. In 1919 and 1920 Harland offered a similar model, with R functioning as a general colour factor and conditioning red in seed coats. In 1956, Smith distinguished three dominant genes, W (Watson eye), H (Holstein patterns) and B (Blood) that regulate the colour of the seed coat of *Vigna sinensis* Savi. The following gene symbols were then assigned to the different cowpea seed coat patterns: *D*, dense speckling; *E*, narrow speckling, *F*, fine and dense speckling; *G*, dotting of Holstein seeds; *S*, spotting (patches of black pigment); *T*, Taylor pattern (thinly scattered bluish dots); *W*, whirlpoolwill mottled pattern (irregular areas of dark shade separated by lighter areas) and *X*, Taylor inhibitor (Spillman & Sando 1930). Fery (1980) later renamed dense speckling *De*; narrow eye, *Na*; dotting, *Dt*; whirlpoolwill, *Wh* and Taylor inhibitor, *In-T*.

Factors such as the rate of imbibition (Powell & Matthews 1978, Powell 1989), germination and ageing (Abdullah *et al.* 1991) have been related to pigmentation in cowpea seeds (Morrison *et al.* 1995). A study by Legesse & Powell (1992) also revealed that cream/beige cultivars showed evidence of imbibition damage indicated by poor vital staining and high rate of solute intake, as compared to coloured seeds with a slow imbibition rate. In other plant species, the colour of the testa has been related to its tannin content (Cabrebra & Martin 1989). According to Werker (1997) colouration of the seed coat may be adapted to the fate of the mature independent seed. The dull brown, blacks and greys, being similar to the soil, may aid in camouflaging the seed and thus protecting it from predators. In other cases, dark pigments in seeds were found to take part in rendering the seed impermeable (Werker 1997). The effect of pathogens on seed colour has also been highlighted in several studies. In a study by Stasz *et al.* (1980), coloured pea seed coats were not penetrated by *Pythium ultimum* 100

hours after planting, whereas, uncoloured seeds were penetrated within 40 hours.

Pigments may be present in the lumen of cells, in cell walls or in both. They can occur at different locations in the cell lumen and/or cell wall, in the outermost layer of palisade cells or sub-palisade cells and the mesophyll (Werker 1997). A combination of various pigments may also occur in the different layers of the seed coat. Pigments may also be situated in the inner layers while the outer layers remain transparent (Harborne 1969). Different structures of the seed may differ in colour from the rest of the seed. The colour of the two is often contrasting, for example, the hilum in most cases is the same as the rest of the seed coat or light in dark seed or dark in light seeds (Werker 1997).

The seed pigmentation in cowpea seed coats is mainly due to anthocyanins located in cells arranged in layers (Burns & Winzer 1962). Fery (1985) referred to cowpea seed pigmentation as melanin-like found only in the seed coat, where it was responsible for a pale yellow to deep copper-red basal colour. This pigment is always present in the third layer and often in the palisade layer of all coloured seed coats. The palisade layers of some coloured seeds also contain anthocyanins and this influence the colour of some plant parts (Werker 1997).

Anthocyanins belong to a group of plant constituents collectively known as flavonoids (Mazza & Miniati 1993). They are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts. Individual anthocyanins are differentiated by the number of hydroxyl groups, the nature and number of sugars in the molecules and the position of the attachment and the nature and number of aliphatic or aromatic acids attached to the sugars in the molecule (Seikel 1962). The naturally known occurring anthocyanidins or aglycones, which occur most frequently in plants, are pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Harborne 1969).

Other chemical constituents in the seed coats are the condensed and hydrolysable tannins. Tannins are high molecular weight phenolic compounds with the ability to form complexes with natural polymers such as dietary protein, digestive enzymes and polysaccharides under suitable pH and concentration (McLeod 1974). Condensed tannins are polyflavonoid in nature, consisting of two or more flavan-3-ol units (such as catechin, epicatechin or corresponding gallocatechin) (Porter 1989). These compounds do not contain a sugar residue and are more resistant to breakage than hydrolysable tannins. Hydrolysable tannins contain a core of a polyhydric, usually glucose, which is esterified to a number of phenolic carboxylic acids (Bernays *et al.* 1989). Condensed tannins are mainly responsible for protecting the plant against microbial and fungal attack, whereas hydrolysable tannins serve to inactivate the digestive enzymes of the herbivore, especially of insects. The combined effect of the tannins would then result in a reduction of fermentative activity and degradation of fibrous tissues (Bernays *et al.* 1989).

There has been no adequate investigation on the influence of seed colour on the resistance of cowpea seeds to the anthracnose pathogen. Dark coloured seeds tend to be more resistant to storage insects than light coloured seeds. This was observed with some of the seeds stored prior to use in this study (Chapter 5). In other grains such as sorghum, dark coloured seeds are known to contain more tannins and are thus more resistant to insect, bird and fungal attack (Price *et al.* 1979). Based on this, it could be hypothesised that dark coloured seeds should show some resistance to *C. dematium.*

2.3 Food uses of cowpea

For centuries, many varieties of cowpea have been cultivated throughout Africa and Asia as food for humans (Singh & Allen 1979). All the parts of the cowpea plant have a specific use at different times of its growth. In fresh form, the young leaves, immature pods and peas are used as a vegetable (Tindal 1983). In most regions, fresh leaves are prepared as a form of spinach, mixed with coconut milk

or fresh cream or groundnuts, or mixed in stews. Sometimes, the leaves are dried and preserved to be prepared in winter when there are no fresh vegetables, or used as a meat substitute (Fox & Young 1982). The grain contains, on average, 21-25% protein and 50-67% starch. After all the parts are utilised for human consumption, the above-ground (or aerial) parts are harvested for fodder. In areas such as west and central Africa the fodder is cut and stored for subsequent sale at the peak of the dry season (Bressani 1985, Quin 1997).

As a vegetable crop, the chemical composition of cowpea (Table 2.1) corresponds with that of most edible legumes (Coetzee 1995). The seeds also contain small amounts of ß-carotene equivalents, thiamin, riboflavin, vitamin A, niacin, folic acid and ascorbic acid (Kay 1979, Tindal 1983). The use of cowpea seeds as a vegetable provides an inexpensive source of protein in the diet. The dried pulse may be cooked together with other vegetables to make a thick soup, or ground into a meal or paste, before preparation in a variety of ways (Kay 1979, Allen 1983, Quass 1995).

Table 2.1
Chemical composition (%) of cowpea (Kay 1979, Tindal 1983, Quass 1995)

	Seeds	**Hay**	**Leaves**
Carbohydrate	56-66	-	8
Protein	22-24	18	4.7
Water	11	9.6	85
Crude fibre	5.9-7.3	23.3	2
Ash	3.4-3.9	11.3	-
Fat	1.3-1.5	2.6	0.3
Phosphorus	0.146	-	0.063
Calcium	0.104-0.076	-	0.256
Iron	0.005	-	0.005

2.4 Cowpea pathogens: *Colletotrichum* species

Colletotrichum is one of the most important genera of pathogenic fungi worldwide, causing economically important disease of cereals, grasses, legumes, vegetables and perennial crops including trees. The fungi attack all parts of the plant, at all stages of development, from seedlings to mature plant and seed, causing disease symptoms commonly known as anthracnose (Bailey & Jeger 1992).

In 1831, Corda characterised the genus *Colletotrichum* as fungi with hyaline, fusiform, curved conidia and setose acervuli. This was based on the discovery of *C. lineola* found on dead stem tissue. According to Sutton 1966, the name was originally spelt as *Colletothricium* by Corda in 1831 but was later changed to its present spelling in 1837. A period of uncertainty followed, whereby the genus *Colletotrichum* could not be distinguished from *Vermicularia* Tode and was considered congeneric by Duke (1928), and from *Gloeosporium* Desm & Mont., originally described as producing glabrous acervuli (Baker *et al.* 1940). Von Arx (1957 & 1970) later used monographs to differentiate between the species. In these studies, several taxa were grouped into 13 accepted species groups, which included about 288 species of *Gloeosporium* as well as some members of *Vermicularia*. Sutton (1962) expressed the opinion that descriptions and classifications by von Arx (1957) were based on inadequate examination of original material and insufficient study of the biology of the species concerned. Subsequent studies by Gorter (1962), Sutton (1968), and Hindorf (1973) investigated the propositions made by von Arx. Lenne (1978) evaluated various approaches to species separation, including isozyme analysis, which served to consolidate the distinctions made using traditional approaches. Sutton (1980) provided a more recent generic diagnosis of *Colletotrichum*.

Colletotrichum species have been known to cause significant losses in cowpea production (Emechebe & Florini 1997). The three major *Colletotrichum* species causing economic losses will be briefly discussed in this review. These are,

Colletotrichum lindemuthianum (Sacc. & Magn.) Briosi & Cav., the *Colletotrichum capsici* (H.Syd) E. Butl and Bisby - *Colletotrichum truncatum* (Schwein.) complex and *Colletotrichum dematium* (Pers.:Fr.) Grove.

2.4.1. *Colletotrichum lindemuthianum*

2.4.1.1 History and morphology

Cowpea anthracnose was first reported in 1985 (Prassana) in India. The anthracnose of common bean caused by *C. lindemuthianum* has been studied widely since 1957 (Hubbeling) and it is one of the most well studied diseases caused by *Colletotrichum*. The bean anthracnose has a worldwide distribution causing severe economic losses throughout the world (Allen 1983, Pastor-Corrales & Tu 1989). At one time, it was considered to be the most important disease in the bean producing area of eastern USA (Tu 1992). Until recently, a form of *C. lindemuthianum* was regarded as the cowpea anthracnose pathogen (Williams 1975). However, on the basis of molecular, morphological and antigen differences that exist between the anthracnose pathogens of cowpea and *Phaseolus* beans, it has been suggested that the cowpea anthracnose pathogen is probably a form of *Colletotrichum gloeosporioides* (Penz) Penz. & Sacc. (Emechebe & Florini 1997). Several races of the species have been reported from America, France, Germany and Australia. In each race there are several different forms, which differ not only in growth rate and colony appearance, but also in conidial production and pathogenicity (Batista & Chaves 1982).

In culture, the colonies of *C. lindemuthianum* appear grey then rapidly turn darker as aerial mycelia become compact, with the reverse almost black. Conidia are formed as salmon to honey-coloured masses, straight, cylindrical to ellipsoid, with both ends obtuse and measuring 9.5–11.5 x 3.5–4.5 µm (Mordue 1971, Baxter & van der Westhuizen 1984). Appressoria are sparse, ranging from cinnamon to dark brown in colour, clavate or ovate with regular edges (Baxter *et al.* 1983).

2.4.1.2 Interaction with hosts

Anthracnose caused by *C. lindemuthianum*, is principally a stem disease, which is partially severe in crops grown in monoculture where it spreads rapidly under wet conditions and at temperatures between 15 and 20°C (O'Connell *et al.* 1985). The disease appears as individual lenticular to circular, brown to tannish pink, sunken lesions with dark red margins. These can develop into large, spreading, dark lesions on the girdle, branches and petiole. Coalescence of lesions leads to chlorosis and leaf death (O'Connell *et al.* 1985).

At least seven races of *Colletotrichum lindemuthianum* have been known since 1977, these are morphologically indistinguishable, and differentiation depends on different hosts (Hubbeling 1976). This race-cultivar interaction is explained when the incompatibility of *C. lindemuthianum* with a particular cultivar of *Phaseolus vulgaris* L. is characterised by early host death at the point of penetration (Esquerre-Tugaye *et al.* 1992). This inhibits further growth of the pathogen in the penetrated tissue and thus limits lesion growth. This early hypersensitive response does not occur in compatible interactions, thereby indicating a high correlation between hypersensitive response and race-cultivar specificity (Esquerre-Tugaye *et al.* 1992). At the cellular level, wall appositions and papilla formation are encountered by the pathogen. These formations, which prevent infection, are produced in response to infection of resistant and susceptible plants (Esquerre-Tugaye *et al.* 1992).

Germination of conidia of *C. lindemuthianum* occurs within 18 h after inoculation on the French bean (O'Connell *et al.* 1985) and 12 h after inoculation on cowpea stems (Bailey *et al.* 1990). The appressoria of this fungus on cowpea are reported to be produced indiscriminately over the entire surface of the host, except over the stomata (Bailey *et al.* 1990).

Epidemiology studies on C. *lindemuthianum* investigating the effect of different temperatures indicated that the disease caused by this pathogen is more severe

at 15-24^{0}C, and significantly reduced at 28-32^{0}C (Williams 1975, Pastor-Corrales & Tu 1989). Disease severity differs significantly between cultivars and different temperatures. In tropical developing countries, bean yields are low and diseases are the principal contributing factor (Emechebe 1981). Yield losses of crops of 90-100% due to anthracnose have been reported in many countries throughout the tropics of Africa (Allen 1983), America (Pastor-Corrales & Tu 1989) and Asia (Buddenhagen *et al.* 1987). Yield losses as high as 50% have been recorded in monocropped fields in the humid regions of Nigeria (Williams 1975). Losses are probably lower on mixed-cropped peasant farms but no quantification of the losses in such production systems has been done (Williams 1975).

2.4.2 Colletotrichum truncatum and *C. capsici*

2.4.2.1. History and morphology

Colletotrichum capsici was once considered to be a synonym of *C. dematium* by von Arx (1957) but later it was placed as a distinct species occurring on *Capsicum* (von Arx 1981). It was differentiated from *C. dematium* because of its wider conidia (Sutton 1980) and because it is pathogenic, whereas, *C. dematium* was once believed to be a saprobe (Mordue 1971). Von Arx (1957) accepted *C. truncatum* as a *forma specialis* of *C. dematium*. Sutton (1980) also distinguished *C. dematium* from *C. truncatum* on the basis of the latter having broader conidia.

The difference between *C. truncatum* and *C. capsici* in culture is that *C. capsici* colonies appear as a dense white to dark grey mass that is dark brown on the reverse side, with abundant setae (Holliday 1980). The conidia form a pale buff to salmon mass. Conidia are falcate, fusiform, gradually tapering towards the ends, and each measuring 18-23 x 3.5 μm. *C. truncatum* has cottony to floccose or oppressed, pale mouse grey to grey or salmon or cinnamon colonies. Setae are sparse and the conidia are formed in saffron to orange masses, falcate, fusiform and tapered gradually towards the obtuse apex but abruptly towards the truncate base and measure 12-16 x 4-6 μm (Holiday 1980, Koch *et al.* 1989).

2.4.2.2. Interaction with hosts

Brown blotch caused by *C. truncatum* and *C. capsici* is one of the diseases of cowpea causing losses greater than 75% in the savannah region of Nigeria (Emechebe 1981). Pring *et al.* (1995) described the symptoms as non-pigmented lesions that rapidly spread to rot the entire tissue. Lesions turn purplish brown and appear as dark brown blotches on stems and pods. The necrosis of the flowering axis accompanied by floral abortion and distortion of immature pods is the most destructive phase of brown blotch disease of cowpeas (Oladiran & Oso 1983). The disease further develops as purplish or reddish brown blotches on petioles, leaf veins and peduncles without the formation of a definite lesion. Girdling results in stem collapse. On pods, sporulation appears as alternating black and brown bands and this is a diagnostic feature of the disease (Emechebe & McDonald 1979).

Colletotrichum truncatum affects many seed and forage legumes (Lenne & Sonoda 1978b), notably soybean (Manandhar *et al.* 1985) and lentil (Gibson *et al.* 1991). The host range of *C. capsici* on legumes includes the following species: *Vigna mungo* L. (Kumar *et al.* 1989), *Medicago sativa* L., *Pisum sativum* L., and *Arachis hypogea* L., (Lenne 1992). Generally, *C. capsici* is regarded as an unspecialised pathogen (Mordue 1971), however, it occurs more frequently on cowpea (Allen 1983). The incidence of seedborne *C. capsici,* as illustrated in most field experiments, indicated that pods with brown blotch often yielded 100% *C. capsici* seed infection, but only 0-7% *C. truncatum* (Emechebe & McDonald 1979). Seed-to-plant transmission was 38% for *C. capsici* and 30% for *C. truncatum*. Therefore *C. capsici* is considered the principal pathogen in the cowpea brown-blotch complex (Emechebe & McDonald 1979).

In disease evaluation studies, *C. truncatum* affects the soybean plant at all stages of development, particularly from bloom to fill, thus making this host one of the most susceptible (Siddiqui *et al.* 1983). In this host-pathogen interaction, symptoms appeared in the early reproductive stages on stems, pods and petioles

as irregularly shaped brown lesions often because of latent infection. The pathogen may survive up to 10 years on soybean and mungbean stored at 5^0C. Because of its high pathogenicity and most frequent occurrence, *C. truncatum* is regarded as the most important seed-borne disease pathogen of legumes (Siddiqui *et al.* 1983). Conidial germination occurs at 16 h for *C. capsici* on cowpea hypocotyls (Bailey *et al.* 1990) and as early as 4 h after inoculation when *C. truncatum* infects pea (O'Connell *et al.* 1993). The brown blotch complex caused by *C. capsici* and *C. truncatum* is more important in the drier savannahs than in humid conditions which are usually favourable for some *Colletotrichum* spp. (Lenne 1992). The anthracnose of soybean caused by *C. truncatum* is more severe in warm moist weather at 20-25^0C (Sinclair & Beckman 1989).

2.4.3 Colletotrichum dematium

2.4.3.1 History and morphology

Fries originally described *Colletotrichum dematium* as *Sphaeria trichella* F. in 1818, then as *Sphaeria dematium* in 1823. In 1862, Fries suggested that papillate *Sphaeria* species, including *S. dematium*, should be placed in *Vermicularia* Tode (1825). Grove (1918) placed *S. dematium* in *Colletotrichum.* A number of species in the genus *Colletotrichum* have been placed under *C. dematium* either as synonyms, variations, forms or *formae speciales*. These classifications were based on morphological features such as conidia and host specificity. These species included *C. spinaciae* Ell. & Halst. accepted as a form that is specific to Spinaceae and *C. truncatum* (Schwein, Adrys & Moore) accepted as a *forma specialis* (von Arx 1981, Holliday 1989, Koch *et al.* 1989). *C. trichellum* (Fr.:Fr.) Duke, which was once considered to be a form of *C. dematium* in 1957 by von Arx was later considered a different species by Sutton in 1962. *C. capsici* was once considered a synonym of *C. dematium* by von Arx (1957) but later placed in a different taxon by Sutton (1980). *C. circinans* (Berk.) Vodino was accepted as being specific to *Allium* spp. under the name *C. dematium* (von Arx 1957, 1981). *C. corchori* Ikata & Tanaka, was suggested to be a form of *C. dematium* by Holliday (1980) and *C. curvatum* Briant. & Martyn

was placed as a synonym of *C. dematium* by von Arx (1957). The most widely used descriptive features of *C. dematium* are that the colonies exhibit a wide range of pigmentation, from white to black grey, with abundant black conical setae, brown appressoria that are clavate or ovate, 7.5-18 x 4-12 µm, with irregular edges. The conidia are abundant, fusiform, 18-26 x 2-3 µm (Sutton 1980), tapering at each end (Baxter *et al.* 1983). These features were used in this study to identify isolates of *C. dematium* collected from cowpea fields.

Many *Colletotrichum* species have been described on Leguminosae, mostly on temperate crops (Lenne 1992). Those with curved or boat-shaped conidia were placed under *C. dematium* and seem more important in the tropics and subtropics (Holliday 1980). Anthracnose caused by *C. dematium* was first recorded on cowpeas in India (1977) and Malaysia (1984) (Lenne 1992). In South Africa, Smith & Aveling (1997) observed the disease in KwaZulu-Natal in 1997 and the symptoms were described as purplish brown, necrotic lesions on the stem and pods. The fungus is seed-borne and may penetrate the seed coat (Sutton 1962; Holliday 1980; Smith & Aveling 1997).

2.4.3.2 Interaction with hosts

The host range of *C. dematium* varies widely but it infects mostly leguminous crops, although it occurs on some non-leguminous hosts. The host range includes table beet (Gourley 1966); soybeans (Roy 1982); prickly sida, spotted spurge and smooth pigweed (McLean & Roy 1988). There have been reports on many other hosts both in the tropics and sub-tropical regions, where it causes minor diseases such as leaf spot and die-back (Ram & Lele 1968). Recently there has been a report of *C. dematium* on ripe fruit of tomato (*Lycopersicon esculentum* Mill. var *pyrifoma* (Dunal) L. H. Bailey) in Argentina (Bello 2000).

In 1951 Tiffany found that some varieties of *C. dematium* were pathogenic on seedlings of soybean. *C. dematium* var. *truncata* caused seed-rot, pre-emergence and post-emergence killing and blighting of soybean seedlings (Tifanny 1951). However, *Colletotrichum dematium* was once described as a pathogen of mature tissue (Sutton 1962) and has been isolated from dead

herbaceous stems or leaves. Gourley (1966) described *C. dematium* as a good coloniser of senescent tissue. Roy (1982) found that *C. dematium* var. *truncata* together with *C. gloeosporioides* and its teleomorph [*Glomerella cingulata* (Stoneman) Spauld & H. Schrenk.], cause severe hypocotyl and cotyledonary infection and significant stunting of seedlings of soybean.

Conidial germination of *C. dematium* on cowpea stems occurs at about 6 h after inoculation. Appressoria are produced over the host's epidermis with no specific orientation to stomata (Smith *et al.* 1999). *C. dematium* enters the host directly through the cuticle and epidermal walls, and it is not reliant on wounds or natural openings for penetration (Smith *et al.* 1999). Anthracnose caused by *C. dematium* causes major economic losses in wet, humid, tropical, forest regions of Africa, Latin America and Asia (Emechebe & Florini 1997) and is not as common in the drier savannahs (Lenne 1992). Likewise, *C. dematium* has been reported in areas such as Argentina (Bello 2000), where conditions are humid as well as areas such as Gauteng (South Africa) (Smith 1997) where conditions range from dry and hot to slightly humid, depending on the season (South African Weather Bureau 2000). During a survey of cowpea fields in Mozambique and Zimbabwe, *C. dematium* was prevalent during the summer season where temperatures ranged from 25-33^{0}C [Pakela, Tombolane & Mabena, 1998 (Internal Report)].

Environmental factors that influence the disease severity of *C. dematium* on cowpea have not been documented. Investigation of the epidemiology of the fungus can be useful in combating the disease. This study will provide useful information on the factors affecting the infection of cowpea by *C. dematium* in the greenhouse. This information can then be compared with the response of the fungus under field conditions.

2.5 Genetic relatedness in *Colletotrichum*

Isolates of *Colletotrichum* are known to vary greatly in morphology, host preference, pathogenicity and physiology, making taxonomic studies difficult. Phylogenic relatedness has been based on host range, developmental and anamorphic characteristics. Increased knowledge of field isolates, however, has made it apparent that there are limitations associated with classifications based solely on morphology, since critical morphological traits may vary greatly and be altered by cultural conditions (Sutton 1980).

2.5.1 Vegetative compatibility

One way of determining genetic relatedness is vegetative compatibility. According to Katan (1998), the term vegetative compatibility refers to the ability of individual strains of the same fungal species to undergo mutual asexual hyphal anastomosis, which results in viable fused cells containing nuclei of both parental strains in a common cytoplasm. Hyphal anastomosis is a common occurrence within a fungal species, but this is not a random phenomenon.

There are few studies of vegetative compatibility groups performed on *Colletotrichum* species. Most *Colletotrichum* species studied are known to be genetically unstable (Brooker *et al.* 1991).

Vegetative compatibility or heterokaryon compatibility has been used in many species of *Fusarium*, mainly to determine the genetic relationship among different strains (Correll *et al.* 1988, Elmer & Stephens 1989). When hyphae of two fungal strains anastomose, they produce a heterokaryon and are then considered to be vegetatively compatible (Elmer & Stephens 1989). Fungi that are vegetatively compatible belong in the same vegetative compatibility group (VCG). Therefore, strains that are in the same VCG are genetically more similar in many traits than vegetatively incompatible strains of the same species (Correll *et al.* 1988). The method for determining VCGs using nitrate non-utilising mutants (*nit* mutants) was developed by Cove in 1976 for *Fusarium* and was later modified by Puhalla (1985). Correll *et al.* (1988) refined the technique by demonstrating that *nit* mutants of *F. oxysporum* could be subdivided into at least

three phenotypic classes (*nit*1, *nit*3 and NitM). These classes could be differentiated by the ability of the *nit* mutants to utilise various nitrogen sources.

Most fungi are able to utilise nitrate as a nitrogen source by reducing it to ammonium via nitrate reductase and nitrite reductase (Garraway & Evans 1984). Chlorate, which is an analogue of nitrogen and useful in nitrate assimilation, is used in this technique. The reduction of chlorate to chlorite by fungi results in chlorate toxicity, i.e. chlorate sensitive fungal strains can reduce nitrate to nitrite but chlorate resistant strains cannot (Correll *et al.* 1987). As a means of adapting to the changing environment, species of *Colletotrichum* form sectors (on chlorate medium) at a relatively high frequency. This, however, indicates that the strains are genetically unstable and because of that *nit* mutants can be readily recovered from field isolates (Brooker *et al.* 1991).

In the process of screening populations for genetic relatedness, two tester strains (strains of known VCG) are made for each VCG. These tester strains are then paired with *nit* mutants derived from field isolates with unknown VCG. When complementation occurs between a known VCG of the tester strain and the field isolates, the field isolate is assigned the VCG of the tester strain. This process depends on the ability of the two strains to form a heterokaryon under the conditions described. Strains, which are unable to form heterokaryons, are designated heterokaryon self-incompatible or vegetatively self-incompatible (Jacobson & Gordon 1988, Correll *et al.* 1989).

In a study by Brooker *et al.* (1991) three Colletotrichum species, namely, *C. gloeosporioides*, *C. malvarum* (Braun & Casp.) South. and *C. trifolii* Bain. & Assary, behaved as heterokaryon self-compatible in that paired mutants from the same parent complemented each other and formed heterokaryons. When mutants from different isolates of the same species were paired, no complementation was observed indicating the existence of different VCGs. Strains of *C. destructivum* and *C. fragariae* A. N. Brooks, were heterokaryon self-

incompatible. Studies of VCGs of *C. gloeosporioides* indicated the existence of at least six VCGs amongst morphologically similar samples of 19 apple isolates from around New Zealand (Skipp *et al.* 1995).

2.5.2 Molecular techniques

Sutton (1992) suggested that relationships within the genus *Colletotrichum* were unlikely to be resolved using morphology alone. To compliment VCGs, which normally represent clonal lineages within species (Koenig *et al.* 1997), genetic diversity among isolates can be further determined using different molecular techniques. Molecular techniques using genetic markers generated by random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990) have been proved to be useful in determining the genetic structure and evaluating relationships amongst *Colletotrichum* populations (Freeman *et al.* 1993, Screenivasaprasad *et al.* 1993). Species identification (Freeman & Katan 1997, Saha *et al.* 2002), determination of genomic and pathogenic diversity (Munaut *et al.* 2002) and strain distinction (Dales *et al.* 1988) are included in the list of analyses by RAPD. Use of ribosomal DNA (rDNA) restriction digest analysis and sequence data as molecular methods for species delineation has been reported in *Colletotrichum* species (Screenivasaprasad *et al.* 1994, Sheriff *et al.* 1995, Freeman *et al.* 1996, Buddie *et al.* 1999). The internal transcriber spacers ITS1 and ITS2 in the ribosomal RNA gene block, displaying high rates of genetic drift, may also be useful for understanding phylogenetic relationships at a sub-generic level (Lee & Taylor 1992, Screenivasaprasad *et al.* 1996, Munaut *et al.* 2002). In the present study, random amplified microsatellite (RAM) first described by Hantula *et al.* (1996) will be used. RAM are based on polymerase chain reaction (PCR) and uses primers containing microsatellite sequences and degenerate anchors at the 5' end. The RAM patterns are said to be highly reproducible and are applicable to all fungal species. According to Zietkiewics *et al.* (1994), the DNA fragments amplified by RAM reaction are composed of two microsatellites located close enough to be amplified in PCR and the area between them. Evolutionary rates within microsatellites are considered higher than in most other types of DNA

(Charlesworth *et al.* 1994), therefore, there is a greater likelihood of finding polymorphisms by RAM than by most other techniques including RAPDs (Hantula *et al.* 1996).

Colletotrichum infects a wide range of legumes, ornamental plants, fruits and vegetables. Its pathogenicity also varies, from parasitic to saprophytic. Due to these factors, the use of morphology-based taxonomic and grouping techniques would prove inadequate for this species. Therefore, vegetative compatibility groups as well as random amplified microsatellites (RAM) will be used to characterise genetic variation within *C. dematium* (Chapter 3).

2.6 Conidiogenesis in *Colletotrichum*

Conidiogenesis is defined as the method of conidium formation (Cole & Samson 1979). Conidiogenous cells in *Colletotrichum* are described as enteroblastic, phialidic, hyaline to brown in colour, smooth textured, cylindrical to sub-cylindrical. They can be integrated or discrete, with a conspicuous colarette and occasionally prominent periclinal thickening (Sutton 1966). Conidiogenous cells are usually aggregated in conidiomata, but may also be formed as side branches of the mycelium in a hyphomycetous manner (Clausen 1912; Blakeman & Hornby 1966).

Studies by Morgan-Jones (1971) who investigated conidium ontogeny in *C. pyrole*, described formation of the first conidium. During conidium formation, the apex of the conidiogenous cell becomes swollen and the conidium is released by a circumcissile break in the periclinal wall, leaving an opening through which conidia are produced in basipetal succession. It was also noted that the bases of the conidia of *C. pyrole* are narrower than the aperture produced.

In 1971, Ellis described conidiogenesis in *Colletotrichum* as phialidic. The following year, Griffiths & Campbell (1972), however, found that the conidiogenous cells of *C. atramentarium* (B. & Br.) Taub. consisted of two layers,

of which the innermost protruded into the lumen of the conidiogenous cell at the apex. This formed a collar, which was forced outwards by cytoplasmic protrusion during conidiogenesis, thus rupturing the cell wall. According to this description, it was concluded that this type of conidium ontogeny could not be termed phialidic unless a third layer was present, as the inner wall of the conidiogenous cell appeared to be involved in conidium formation (Ellis 1971).

Cole & Samson (1979) later revised the concept of phialidic development of conidia. They stated that the formation of phialoconidia occurs by extension growth of the inner phialide wall, after holoblastic formation of the primary conidium and the rupture of the outer phialide wall. As each conidium secedes, this inner wall ruptures, leaving a ring of torn wall material, which results in the apical thickening characteristic of phialidic development. Baxter (1981) found that conidium ontogeny was constantly phialidic with occasional percurrent proliferation in some species of *Colletotrichum*. Illustrated diagrams of the structural morphology of conidiogenous cells and conidia of *C. dematium* are presented in Fig. 2.1 (Baxter 1981).

Structural studies of *Colletotrichum* species by Sutton (1966) revealed two distinct types of acervuli. The pulvinate acervulus is formed within an epidermal cell and depends on the pathogen to use mechanical force to rupture the epidermal wall and then tear the cuticle. In this case, the hyphae aggregate within the epidermal cells and adjacent parenchyma, rupturing the lateral walls and subsequently the outer wall and cuticle. The latter are pushed aside as the setae, conidiophores and pseudoparenchymatous stroma develop. The pulvinate acervulus is the most common in most *Colletotrichum* species. The second is the hypostromatic acervulus, which is a feature of *C. graminicola* (Ces.) Wilson and other species that attack graminaceous plants. As in pulvinate development, hyphae develop in the epidermal cells, but they produce penetration hyphae, which grow through the outer epidermal cell walls and form conidiophores and setal initials between these and the cuticle. As the initials increase in size, the cuticle ruptures (Sutton 1966).

Conidia of *Colletotrichum* are hyaline, aseptate, sometimes becoming uniseptate prior to germination, smooth-textured, straight or falcate, cylindrical to fusiform (Sutton 1966). The conidia collect in a mucilaginous fibrillar material (Griffiths & Campbell 1972). This is composed of polysaccharides and proteins and is water-soluble. Suggested roles for this matrix are that it protects conidia against desiccation and increases efficiency of germination and host penetration through the activity of the invertase and hydrolase which it contains (Nicholson & Moraes 1980).

Baxter (1981) has briefly discussed some aspects of conidiogenesis of some *Colletotrichum* species, including *C. dematium*. This study will focus on the ultrastructure of the development of conidiogenous cells, conidiophores, conidia and acervuli (Chapter 4).

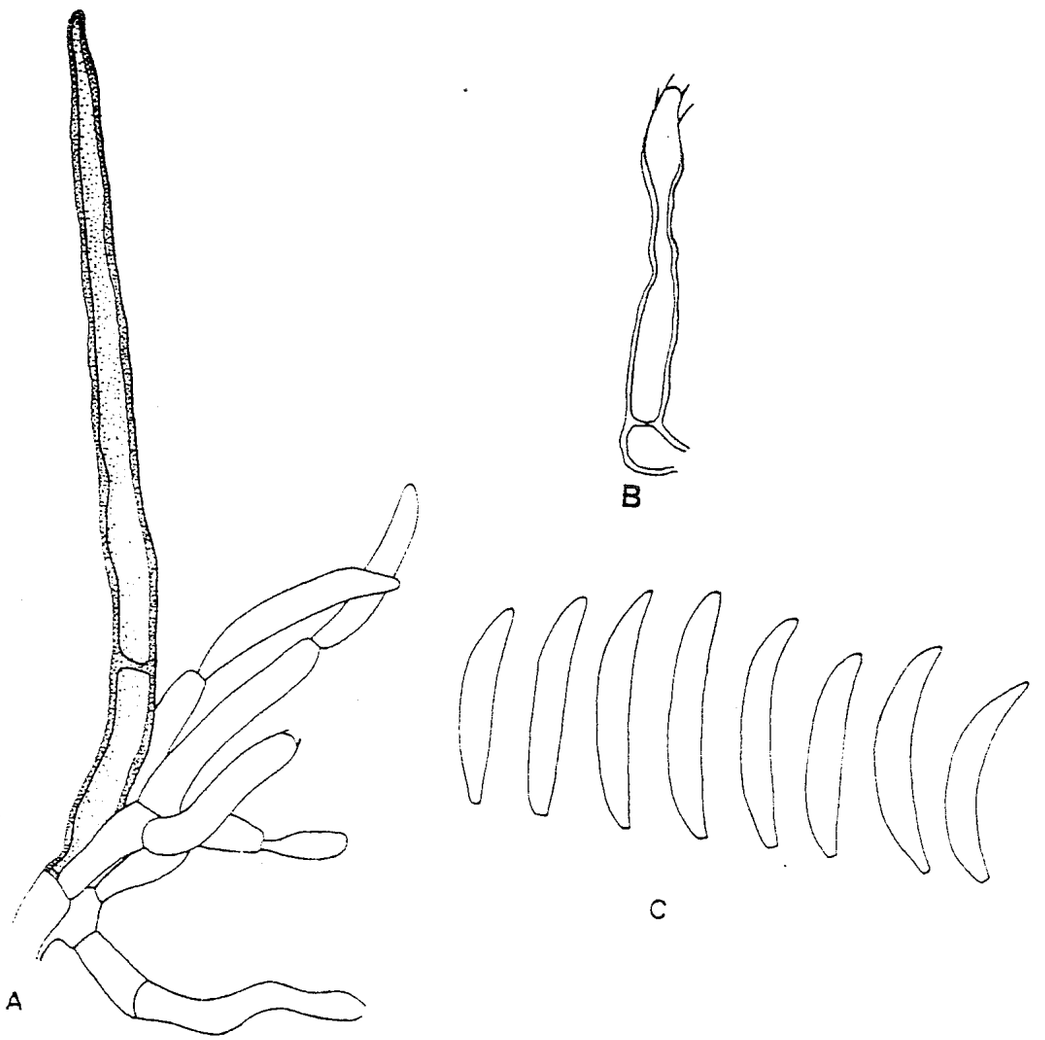

Figure 2.1

Line illustrations of conidiogenesis of *C. dematium* (Baxter 1981). A. Seta and conidiogenous cells; B. Proliferating conidiogenous cell; C. Conidia.

2. 7 Infection strategies of *Colletotrichum* species

Colletotrichum species are among the most successful plant pathogenic fungi exhibiting three main initial infection strategies, according to which species are loosely categorised (Bailey *et al.* 1992). The infection processes of several *Colletotrichum* species such as *C. lindemuthianum* (O'Connell *et al.* 1985), *C. destructivum* (Latunde-Dada *et al.* 1996), *C. truncatum* (O'Connell *et al.* 1993) and to some extent *C. dematium* (Smith *et al.* 1999), have been well characterised by light and electron microscopy. The typical infection process of *Colletotrichum* involves a common sequence of events as described by Dodd *et al.* (1989). The inoculum arrives at the host's surface as water- or splash-borne conidia, which become attached to the plant cuticle. Conidia become septate prior to germination usually within 12-48 h after inoculation. A germ tube emerges and grows for about 10-20 µm before terminating in an appressorium. Appressorium shapes are highly characteristic of the genus, and they become vacuolated and septate (Emmert & Parbery 1975), globose to oval and irregular in outline with varying degrees of lobbing (Lenne 1978). As the appressoria age, they become darkly pigmented with melanin (Jeffries *et al.* 1990).

The events of the infection cycle of *Colletotrichum* species are similar with slight variations. In some cases, the appressoria may go through a stage of extended dormancy before forming an infection peg. The infection peg grows through the cuticle to the epidermal cell. Infecting hyphae spread both inter- and intracellulary as lesions develop (Jeffries *et al.* 1990). There are differences in the duration as well as styles of initial penetration and infection processes of the species. These morphological event variations are listed in Fig. 2.2 (O'Connell *et al.* 1985, Jeffries *et al.* 1990, van Dyke & Mims 1991, Smith *et al.* 1999).

Infection strategies in *Colletotrichum* seem to incorporate several key elements of pathogenesis. Firstly, an essential characteristic of invasion of host cells intracellularly, the biotrophic or hemibiotrophic phase, without triggering hypersensitive reaction, is an initial avoidance of cell death (Jeffries *et al.* 1990).

Secondly, the pathogen seems to influence the timing and intensity of cell degeneration to create a cellular environment suitable for rapid fungal growth during the necrotrophic phase (Jeffries *et al.* 1990). There is no evidence on whether the initial degeneration of infected host cells occurs by autolysis resulting from accelerated senescence or by direct action of the fungal enzymes or toxins. The third aspect is that, during initial infection, the pathogen achieves minimal stimulation of biosynthesis of phytoalexins and other plant defence compounds in the surrounding tissue (O'Connell *et al.* 1985, O'Connell & Bailey 1986, Mould *et al.* 1991a & b).

2.7.1 Penetration

Penetration mechanisms of *Colletotrichum* species vary widely from penetration through stomata, hydathodes, and wounds to direct penetration of the cuticle. In some cases where the epidermal layer is attacked by insects or mechanical injury or after fruit ripening, the pathogen is able to colonise and kill the injured tissue (Bailey *et al.* 1992). However, for the majority of *Colletotrichum* species, direct penetration of the cuticle mechanically or by secretion of cutinase, or both, is followed by the formation of an appressorium and an infection peg that initiates infection (Bailey *et al.* 1992).

The melanin in the appressorium gives them their typical dark colour and is characteristic of most *Colletotrichum* spp. Melanin plays a crucial role in protection from harmful irradiation and in the penetration of the host epidermis. According to Kubo *et al.* (1982), melanin strengthens the appressorial wall so that it can support the internal hydrostatic pressure necessary for penetration of the cuticle and thus determines the direction of the emergence of the penetration peg. This was supported by Howard & Ferrari (1989) who considered that the primary role of melanin was to trap solutes inside the appressoria. This causes absorption of water by osmosis and in turn creates internal hydrostatic pressure. The pressure creates the mechanical force necessary to penetrate the cuticle directly.

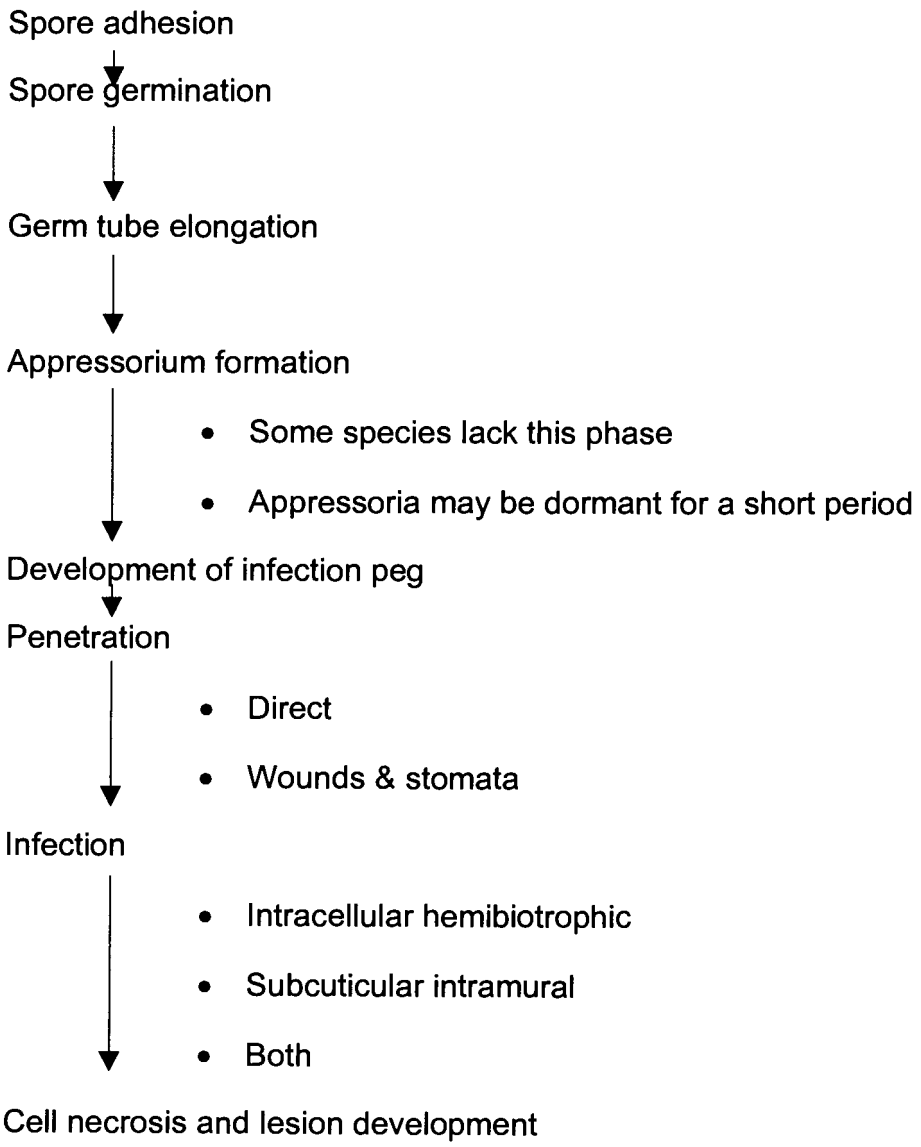

Figure 2.2

Morphological events in the infection process of *Colletotrichum* species (adapted from O'Connell *et al.* 1985, Jeffries *et al.* 1990, van Dyke & Mims 1991, Smith *et al.* 1999.

Enzymes are also produced to dissolve or soften the host cuticle during penetration. Esterase is one such enzyme capable of degrading cutin and is produced by several species of *Colletotrichum*. In a study by Dickman *et al.* (1982), *C. gloeosporioides* and *C. capsici* produced cutinases when grown in liquid culture. In a subsequent study, it was shown that cutinase-deficient mutants of *C. gloeosporioides* were not pathogenic when placed on surfaces of intact papaya fruits, but when fruit were wounded or treated with cutinase, lesions developed (Dickman & Patil 1986).

2.7.2 Enzymes involved in the infection process of *Colletotrichum*

Two groups of enzymes are thought to play an important role in the infection process of pathogenic fungi. The first group is considered to influence the establishment of infection and maceration of the tissue and the second group is mainly concerned with pathogen nutrition (Anderson 1978; Wijesurendera *et al.* 1989). In the first point of interaction between the host and pathogen, cutinase is necessary for penetration for infection to proceed on papaya fruits infected with *C. gloeosporioides* (Dickman *et al.* 1982). This was later confirmed by Dickman & Patil (1986) when cutinase-deficient mutants were unable to penetrate and infect unwounded papaya fruits. The mutants however, were able to produce lesions on wounded fruits or when they were treated with purified *C. gloeosporioides* cutinase prior to inoculation. The importance of cutinase was also observed in other *Colletotrichum* species such as *C. graminicola* on maize seedlings (Kollattukudy 1989), *C. lindemuthianum* on cucumber (Bonnen & Hammerschmidt 1989) and *C. obiculare*, which produces two types of cutinolytic enzymes on cucumber (Bonnen & Hammerschmidt 1989).

Following penetration, for successful infection to proceed the periclinal walls must be penetrated. *Colletotrichum* species are thought to grow through these walls and other cell walls through the secretion of cell wall degrading enzymes such as polygalacturonases and pectinases. In addition, low molecular weight phytotoxins and secondary metabolites such as cellopyrones, colletotrichins and

aspergillo-marasmins (Grove *et al.* 1966, Balio *et al.* 1969) are also believed to be involved. Polygalacturonases, a class of cell wall degrading enzymes produced by *Colletotrichum,* are the first enzymes produced by the fungi when cultured artificially in the presence of plant cell walls (Keon *et al.* 1990). Pectin lyase is another cell wall degrading enzyme that has been found in the extracellular conidial matrix of *C. orbiculare* (McRae & Stevens 1990) and *C. gloeosporioides* (Louis & Cooke 1985).

2.7.3 Initial infection

Many *Colletotrichum* species exhibit two phases of the infection process. The initial stage involves a symptomless phase during which the pathogen establishes itself within the host. This phase is of two types. The first is the intracellular hemibiotrophic phase whereby the hyphae proliferate within the host cells. This is exhibited by *C. lindemuthianum* on *Phaseolus* and *Vigna* and *C. truncatum* on *Pisum* (O'Connell *et al.* 1985*).* The two-phase infection *of C. lindemuthianum* on bean was first noted by Leach in 1922 who also distinguished between the production of initial primary mycelium and the secondary mycelium. Other *Colletotrichum* species exhibiting this form of initial infection include *C. graminicola* on maize (Politis & Wheeler 1973); *C. truncatum* on pea (Uronu 1989); *C. gloeosporioides* on *Citrus* sp. (Brown 1977). An example of this form of infection is shown by *C. lindemuthianum* on *Phaseolus vulgaris* (O'Connell *et al.* 1985). In this study, infected epidermal cells were invaginated around infection vesicles while the cytoplasm of these cells did not show any structural abnormalities. Bailey (1991) later argued that the survival of infected cells determines the ability of this fungus to grow within the host tissue, because processes such as hypersensitive reaction or cell death could induce plant defence mechanisms that would inhibit further infection. Primary hyphae grow from the infection vesicles into adjacent epidermal and cortical cells and later on the host cytoplasm degenerates quickly. During the period between 24-48 h after initial infection, the infected cells retain their normal ultrastructure and membrane function, later they show osmotic disturbance and the host membrane

degenerates 3-5 d after inoculation (O'Connell *et al.* 1985). However, it should be noted that not all *Colletotrichum* species behave this way. *C. lindemuthianum* on cowpea does not follow this progression, as noted by Bailey *et al.* (1990). In this infection process, the fungus infected living cells and the biotrophic phase does not progress to adjacent cells. This is also described for *C. truncatum* on pea (Uronu 1989). The initial infection of lentil by C. *truncatum* is characterised by a symptomless phase lasting 3 d (Chongo *et al.* 2002).

The second type of the initial phase is the subcuticular intramural phase where the pathogen grows exclusively beneath the cuticle and within the periclinal walls of the epidermal wall. Hyphae do not enter the lumen of the underlying epidermal cells (Walker 1921, O'Connell *et al.* 1993). *Colletotrichum capsici* on *Vigna* and *Gossypium* exhibits a subcuticular intramural infection strategy (Pring *et al.* 1995). When the plant tissue has been successfully colonised, whether by intracellular or intramural infections, pathogen growth switches to classic necrotrophic behaviour (Wijesundera *et al.* 1989). Walker (1921) first described this mode of infection for *C. circinans* on onions. Other studies of *C. capsici* on cowpea (Emechebe 1981) and on cotton (Roberts & Snow 1990) showed similar patterns of infection. In these studies, penetration of the cuticle is followed immediately by dissolution of underlying periclinal cell walls. Like the previous phase, during the first 24 hours after infection, there was no visible evidence of infection, but soon afterwards hyphae grew into the cell walls and cortical cells. Therefore, like in the previous stage, the pathogens avoided triggering a hypersensitive reaction. Some species exhibit both intramural and intracellular infection strategies within the same tissue. Examples include, *C. gloeosporioides* on citrus (Brown 1977) and on *Stylosanthes* (Irwin *et al.* 1984).

2.7.4 Necrotrophic infection

The impending end to the transient biotrophic phase of the infection process of *Colletotrichum* species is signalled by intracellular changes such as an increase in mass, but a decrease in density of cytoplasm, dilation of the endoplasmic reticulum and chloroplast lamellae and an increase in fragility and permeability of the plasma membrane (O'Connell *et al.* 1985). Following these changes are cessation of cytoplasmic streaming, rupture of the tonoplast and plasma membrane and cytoplasmic disorganisation, all these being indicative of cell death (O'Connell *et al.* 1985). The appearance, expansion and darkening of anthracnose lesions occur as secondary hyphae develop as branches from the primary mycelium in dead cells. This phase is associated with the abundant production of cell wall degrading enzymes capable of killing and releasing nutrients in tissues in advance of those containing hyphae (Bailey *et al.* 1992).

The necrotrophic phase is responsible for the anthracnose and blight symptoms that are typical of diseases caused by *Colletotrichum* species (Onesirosan & Barker 1971, Emechebe 1981). Despite the widespread destruction of the tissues, the most important feature of the necrotrophic phase is that the cuticle remains intact. The final stage is the production of acervuli, the fruiting bodies of the pathogen. Production of acervuli and perithecia on plant surfaces seems to require an intact cuticle (Sutton 1962).

Smith *et al.* (1999) did infection studies on *C. dematium* on cowpea stem. This study was on the biotrophic phase of the fungus. There has been no previous study on the infection process of *C. dematium* on cowpea. The present study is on the necrotrophic infection strategy of *C. dematium* on cowpea (Chapter 4).

2.8 Control of *Colletotrichum* on cowpea

Cowpea varieties resistant to anthracnose caused by *C. lindemuthianum* are commercially available (Emechebe & Florini 1997). As the pathogen is seed-borne in cowpea (Emechebe & McDonald 1979), the use of seeds obtained from anthracnose-free multiplication fields is usually combined with the growing of resistant varieties to control the disease (Emechebe & Florini 1997). Some foliar fungicides such as benomyl and carbendazim have reduced losses due to anthracnose, but strains of the fungus resistant to many of the effective fungicides have been detected (Emechebe & Florini 1997). Foliar-applied fungicides are effective under field conditions, but may not be economically feasible for the small-scale farmer (Emechebe & Florini 1997). A study by Smith (1997) on control measures of *C. dematium* on cowpea indicated that captab, thiram, imazil / iprodione and mancozeb are non-systemic (protective) fungicides that prevented growth of *C. dematium* on the external surface of seeds. This inhibited further infection of the seeds.

Biocontrol methods for controlling *Colletotrichum* species on some crops include the use of antagonistic *Bacillus* species which control conidial germination, and antagonistic yeasts which reduce lesion density and necrosis due to *C. graminicola* (Jeffries *et al.* 1990). Biocontrol of brown blotch of cowpea, caused by *C. truncatum,* with *Trichoderma viride* Pers. Ex. Fr. involves drenching the soil with a spore suspension of *T. viride*. This was shown to effectively reduce *C. truncatum* in seeds (Bankole & Adebanjo 1996).

2.9 Screening for resistant cultivars

Screening is one of the important processes involved in breeding programmes of dry beans. Screening aims to ensure that cultivars chosen exhibit increased resistance to a wide range of diseases and insects, better tolerance to environmental stress, increased nitrogen-fixing capacity, better seed quality and improved efficiency in the utilisation of limited soil nutrients (Watt *et al.* 1985). Cowpea improvement programmes in Africa have received attention from 1970 at the International Institute of Tropical Agriculture (Singh & Ntare 1985).

Several methods normally used in the screening process involve different inoculation methods. Reliable inoculation techniques contribute to the ease of screening (Tu & Aylesworth 1980). In the screening of plant parts, inoculation methods such as injecting the inoculum, drilling a hole into the stem, placing inoculum meal or cotton swab dusted with inoculum, wrapping the inoculum around a particular plant tissue, spraying and brushing with a spore suspension are common practices (Zummo & Scott, 1985; 1989; Klapproth & Hawk 1991; Adebitan & Ikotun 1992). In a study by Latunde-Dada (1990) on the assessment of anthracnose in cowpea caused by *C. lindemuthianum,* the reaction on inoculated hypocotyl segments corresponded with the reaction on adult plants.
In all the cases, different reactions were obtained; for example, wrapping the inoculum around a wounded tissue resulted in severe infection because conditions were optimum for infection (Adebitan & Ikotun 1992), whereas injecting the inoculum resulted in confining it (Adebitan & Ikotun 1992). Confinement of inoculum could give false results because the intensity of the inoculum could overcome certain types of resistance (Ullstrup 1949, Klapproth & Hawk 1991). Comparing spraying the inoculum to wrapping and injecting the inoculum on the tissue, the latter is more time consuming (Klapproth & Hawk 1991). Depositing the propagules in one place can result in competition for space, germination and growth development thus reducing the effectiveness of the inoculum (Adebitan & Ikotun 1992).

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CHAPTER 3

MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF *COLLETOTRICHUM DEMATIUM* ISOLATES FROM COWPEA (*VIGNA UNGUICULATA*)

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3.1 Abstract

Colletotrichum dematium, the fungus causing anthracnose on cowpea, was first detected in South African fields of this crop in 1997. In a survey to determine the distribution of this fungus, forty-eight field isolates were collected from different regions of southern Africa. Isolates were compared phenotypically and genotypically. To determine the nature and extent of variation, isolates were examined for colony colour, mycelium growth patterns, conidium and appressorium morphology, pathogenicity, vegetative compatibility and genetic variability by randomly amplified microsatellite polymerase chain reaction (RAMs-PCR) analysis. Morphological variation exhibited by *C. dematium* resulted in 12 categories with isolates varying in colony colour, zonation, sectoring and conidium mass colour. Pathogenicity tests indicated that most isolates from Gauteng were highly pathogenic while isolates from Zimbabwe were non-pathogenic. Of the 48 isolates, 30 were able to form heterokaryons and these could be placed into three vegetative compatibility groups (VCGs). Analysis of *C. dematium* isolates by RAMs-PCR revealed 11 clusters within the population. RAMs-PCR analysis consistently grouped isolates together from the same

geographic region, while some clusters contained isolates that had the same pathogenicity level and belonged to the same VCG.

Keywords: anthracnose, *Colletotrichum dematium*, cowpea, pathogenicity, random amplified microsatellites, vegetative compatibility.

3.2 Introduction

The genus *Colletotrichum* Corda contains important plant pathogens which cause economically significant diseases of legumes, cereals, grasses, vegetables and perennial crops world-wide (William, 1975). *Colletotrichum dematium* (Pers. ex Fr.) Grove causes anthracnose on a broad range of host plants within the Family Fabaceae (legumes) and non-legumes such as onion, brinjal and ornamental flowering plants (McLean and Roy, 1988; Lenne, 1992). In South Africa, *C. dematium* was discovered for the first time on cowpea (*Vigna unguiculata* [L.] Walp) in KwaZulu-Natal in 1997 (Smith & Aveling). On cowpea, *C. dematium* poses a serious threat to the consumer of this crop especially in the developing world, where grains and leaves are an important source of protein (Coetzee, 1995).

Historically, *C. dematium* has been described as having many morphological variants and this has led to this fungus being placed in different groups or *formae speciales* in the genus *Colletotrichum* (von Arx, 1957; Sutton, 1966; Baxter *et al.*, 1983). In 1992 Sutton suggested that relationships within the genus *Colletotrichum* were unlikely to be resolved using morphology alone. Morphological plasticity and overlapping phenotypes is common in *Colletotrichum* and this makes traditional taxonomic criteria unreliable for the accurate delineation of the species.

The use of vegetative compatibility groups subdivide *Colletotrichum* populations into groups that can exchange genetic information via heterokaryosis (Brooker *et al.*, 1991). These studies are sometimes complicated by pathogenicity (Correll *et*

al., 1987), cross-VCG compatibility (Correll *et al.,* 1993) and low occurrence of VCGs in *Colletotrichum* (Brooker *et al.*, 1991). On the other hand, genetic markers generated by random amplified polymorphic DNA (RAPDs) (William *et al.* 1990) have proved to be useful in determining the genetic structure and evaluating relationships amongst *Colletotrichum* populations (Screenivasaprasad *et al.* 1994). Genomic and pathogenic diversity in *Colletotrichum* (Munaut *et al.*, 2002), species identification (Screenivasaprasad *et al.*, 1996a; Freeman & Katan, 1998; Saha *et al.*, 2002) have been determined with the use of RAPD analysis. Other molecular methods that have been used successfully in characterisation of *Colletotrichum* species include restriction fragment length polymorphisms (RFLPs) (Buddie *et al.*, 1999; Saha *et al.,* 2002) and internal transcribed spacers ITS1 and ITS2 for understanding phylogenetic relationships at sub-generic levels (Sherrif *et al.*, 1994; Screenivasaprasad *et al.*, 1996b).

The objective of this investigation was to determine relationships between the isolates of *C. dematium* by cultural characterisation, pathogenicity, vegetative compatibility groups and random amplified microsatellite based polymerase chain reaction analysis.

3.3 Materials and Methods

3.3.1 Fungal strains

Forty-eight isolates of *C. dematium* were collected from infected cowpea fields in South Africa [KwaZulu-Natal (KZN) and Gauteng (Field1, Field2 & Field3)], Mozambique (Maputo) and Zimbabwe (Chiredzi). Stem tissue (25mm^2) was surface-sterilised in 1% (v/v) sodium hypochlorite for 2 min, rinsed in sterile water, placed onto water agar and incubated at 25°C. Fungi resembling *C. dematium* were purified and single-spore cultures made by subculturing on potato dextrose agar (PDA) (DIFCO). Identification of cultures was based on spore morphology (Baxter *et al.,* 1983) using a bright field and differential

interference contrast illumination. Single-spore cultures were stored in 15% (v/v) glycerol at –70°C.

3.3.2 Cultural characterisation

Cultural characteristics of 48 isolates of *C. dematium* were compared in 90-mm diameter potato dextrose agar (PDA) and fungal morphology on potato carrot agar (PCA) plates. Plates were incubated at 23°C under 12 h/d irradiation. Appressorium, acervulus and conidium characteristics of these isolates were examined on PCA every eight hours. Slide preparations from 10-d-old cultures were made in the mounting medium polyvinyl alcohol lactic acid glycerine (PVGL) (Koske & Tessier 1983). A bright field and differential interference contrast illumination was used to view the slides. Texture of aerial mycelia, nature of colony edges, and colony zonation were described using the terminology of Hawksworth *et al.* (1995). Colony colour was described according to Rayner's (1970) colour standards.

3.3.3 Pathogenicity tests

Pathogenicity tests were carried out under laboratory conditions. Cowpea seeds *cv.* Rhino, selected for susceptibility to *C. dematium* (Pakela & Aveling, unpublished data), were obtained from the Agricultural Research Council, Grain Crops Institute, Potchefstroom, South Africa. Seeds were surface-sterilised in 1% (v/v) sodium hypochlorite solution for 5 min and rinsed three times in sterile distilled water. Seeds were spread in three replicates of 30 seeds on wet (60 ml sterile water) 305 X 560 mm absorbent cellulose wadding (B.P.C. Agricol Seed, South Africa), backed with two layers of 305 X 560 mm germination paper. The papers were rolled up and secured with rubber band, inserted into plastic bags and incubated in an upright position at room temperature (*ca* 22°C) for 14 days. The hypocotyls of germinated seedlings were cut into 5 cm long sections. Each section was wounded at the centre with a sterile needle. A piece (4-9 mm^2) of 5-day-old *C. dematium* culture was placed beside the wound. Culture pieces were

omitted from controls. Inoculated and control hypocotyl sections were placed in humidity chambers and incubated at 26°C. After four days, symptoms that developed were rated on a scale of: 0=no lesion, 1=lesion concentrated around point of infection, 2=lesion spreading from point of inoculation to above 50% of the 5 cm hypocotyl section, and 3=lesion spread over hypocotyl section. Disease indices for each isolate were calculated and ranked using Gupta's Statistical Test.

3.3.4 Vegetative compatibility tests

The procedure described by Brooker *et al.* (1991) was used to determine the vegetative compatibility groups (VCG) of the isolates. Single-spore isolates of *C. dematium* on PDA were grown on 2.5% and 3% (w/v) chlorate medium, and incubated at 25°C for 7-14 days. Fast growing sectors of the fungus that emerged from the chlorate medium were transferred to minimal medium. Thin sparse growth on minimal medium was considered a *nit* mutant. To identify the *nit* mutant physiological types, they were further grown on differential media containing different nitrogen sources. These included hypoxanthine, nitrate, nitrite, ammonium and uric acid. Phenotypic *nit* mutants were stored on minimal medium agar slants at 4°C.

Complementation between all *nit* mutants obtained from isolates collected were tested on minimal media in 90 mm diameter Petri dishes. Two mutants were inoculated 1 cm apart on each plate and the plates were incubated at 23°C for 7-14 days. Complementation was detected by the formation of dense aerial mycelia where two *nit* mutants met and formed a heterokaryon. Non-complementation was detected as overlapping or formation of a barrier where the two mycelia met. This meant that the two *nit* mutants were vegetatively incompatible. Mutants recovered from each isolate were paired with one another in all possible pairwise combinations.

3.3.5 DNA isolation

DNA was isolated from all *C. dematium* isolates by grinding conidia and mycelia in extraction buffer (200 mM Tris-HCl (pH 8) containing 150 mM NaCl, 25 mM EDTA and 0.5% SDS) using a plastic pestle and 1.5 ml Eppendorf tubes. The tubes and their contents were placed in liquid nitrogen for *ca* 1 min, followed immediately by 5 min in boiling water. This was followed by three phenol-chloroform (1:1 v/v) extractions. The DNA was precipitated with 0.1 µl 3 M Na-acetate (pH 7.4) and 0.6 µl ice-cold isopropanol followed by centrifugation at 10 000 x*g* for 20 min. The DNA pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in 100 µl sterile distilled water.

3.3.6 RAMs - PCR analysis

Genomic DNA from isolates was used in PCR reactions containing RAMS primers 5' DHB $(CGA)_5$, 5' DDB$(CCA)_5$ and 5' HBD$(GACA)_4$ (Lieckfield *et al.*, 1993; Buscot *et al.*, 1996). Each PCR reaction contained 2 ng DNA, 200 µM of each dNTP, 600 nM primer, 3.5 units *Taq* DNA polymerase and 1x buffer with $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA). The reaction volume was adjusted to 25 µl using sterile distilled water. PCR conditions consisted of a 2 min denaturation step at 95°C followed by 35 cycles of 30 s at 95°C, 45 s at 48°C and 2 min at 72°C. A final step at 72°C for 7 min completed the reaction. PCR products were visualised on a 1.8% (w/v) agarose gel containing ethidium bromide, under UV illumination. Samples were further separated on a polyacrylamide gel (6% (w/v) acrylamide in 50 mM Tris-Borate-EDTA buffer, 4 h at 9.5 V/cm) and subsequently silver stained (Bassam & Caetano-Anolles, 1993) to visualise amplification products.

3.3.7 Cluster analysis

The PCR profiles produced by the combinations of the three primer sets were scored for the presence or absence of bands between molecular sizes of 200 and 1000 bp for each isolate. The resulting matrix was analysed using the

PAUP* 4.0b10 computer programme and a Neighbour-joining distance tree was computed. Genotypic diversity was calculated according to Stoddart and Taylor (1988).

3.4 Results

3.4.1 Morphological characterisation

The isolates on PDA were grouped according to colony characteristics (Table 3.1). Twelve groups were identified, with the majority of the isolates occurring in groups 1 and 2. The colony colours of these groups varied from pale mouse grey to dark mouse grey, and the conidial mass colour was salmon. Colony zonation ranged from slight to prominent. Sectoring also varied from none to slight, or occasionally.

Based on the morphology on PCA, the isolates of *C. dematium* were characterised by long and well-developed black or dark brown setae radiating from a point in the acervulus. Appressoria were clavate, lobed and frequently formed chains. The colour of the appressoria varied from light brown to dark brown depending on the age of the appressorium. Among the groups, no variation in size, outline or conformation of appressoria was recorded although groups 6 and 9 did not produce any appressoria. Conidia were fusiform and abundant on each culture plate, except for isolate Cd015, which did not produce any conidia. There was no variation in the shape of conidia of the different isolates and their length fell within the range of 19-25 µm.

3.4.2 Pathogenicity tests

Symptoms varied from small dark brown spots to elongated lesions and in some cases the entire hypocotyl piece became water-soaked and covered with acervuli on the third day after inoculation. Of the 48 isolates tested only 3 were non-pathogenic. Isolates differed significantly in aggressiveness and they were

grouped into 4 distinct groups (Table 3.2). Generally, isolates from Gauteng were more pathogenic than isolates from Zimbabwe.

3.4.3 Vegetative compatibility tests

Nit mutants 1, 3 and M were produced in abundance by most *C. dematium* isolates and few isolates produced *nit*2. Complementation was evident after 10-14 days incubation. Complementation occurred between 30 of the 48 isolates of *C. dematium* and the remaining isolates did not form complementation with any other isolate. Isolate Cd012 became degenerate. Heavy wild type growth, medium growth and thin growth were observed when two mutants formed a heterokaryon (Fig 3.1). Based on these growth patterns, the isolates of *C. dematium* were classified into three vegetative compatibility groups. VCG1 comprised 18 isolates from Gauteng and one from Mozambique, VCG2 comprised five isolates from Gauteng, three from Zimbabwe and one from Kwa-Zulu Natal and VCG3 had two isolates from Gauteng (Table 3.3). Isolates Cd006, Cd019 Cd049 and Cd050 formed weak heterokaryons with isolates in VCG 2 and VCG3.

3.4.4 RAMs - PCR Analysis

The three RAMs primers revealed different PCR profiles for each isolate. The three primers were combined in all possible pair-wise combinations and a total of 560 PCR fragments were amplified, ranging in size from 50 bp to 1500 bp. However, only bands between 200 bp and 850 bp were analysed. Bands representing molecular sizes out of this range were not scored in the analysis because they could not be consistently amplified. The data points obtained from bands between 200 bp and 850 bp were sufficient to determine the genetic relationships among the 48 fungal isolates.

A neighbour-joining consensus tree (Fig 3.2) produced from combined RAMs-PCR profiles from three primer combinations was used to assess relatedness

among isolates. There was a high level of variation among the isolates and eleven clusters were observed. Some isolates however, fell outside the 11 clusters observed. The clusters were compared according to RAMs grouping, vegetative compatibility grouping and geographic origin (Table 3.3). Calculated genotypic diversity of the population was 13.25 %.

3.5 Discussion

In this study, the cultural and morphological features among isolates of *C. dematium* confirmed some of those described by Baxter *et al.* (1983) and Koch *et al.* (1988). *C. dematium* isolates showed great diversity in cultural characteristics, even those collected from the same field. Morphological diversity within species of *Colletotrichum* is fairly common and this has led to taxonomic confusion in the past. The similarities of characteristic features, such as appressoria and curved conidia of *C. dematium,* to other *Colletotrichum* species such as *C. truncatum*, *C. circinans* and *C. capsici* has led to these species being grouped together as *formae speciales* by von Arx (1970). In 1980, Sutton on the other hand divided *C. dematium* into five species that included *C. truncatum* and *C. capsici.* It is clear that the classification of *Colletotrichum* species is a controversial issue, with species neither conforming to morphology, host or geographic origin.

Although all the isolates of *C. dematium* used in this study were from the same host the only consistent similarity among the isolates was the shape and size of their conidia, which is also used as a reliable taxonomic tool to differentiate *C. dematium* from other *Colletotrichum* species. Therefore, it can be concluded that colony characteristics among isolates of *C. dematium* vary significantly. In *C. lindemuthianum,* isolates causing anthracnose of cowpea were found to be morphologically distinct from those causing anthracnose on bean (Williams, 1975; Bailey *et al.,* 1990). These differences in *C. lindemuthianum* have resulted in the creation of *formae speciales* (Bailey *et al.,* 1990), which also represented different pathogencity groups.

Isolates of *C. dematium* on cowpea differed significantly in pathogenicity with isolates found in Gauteng varying from non-pathogenic to highly pathogenic while those from Zimbabwe were non-pathogenic to slightly pathogenic. In some *Fusarium* species pathogenicity was used to differentiate strains (Correll, 1991), although there are some inherent problems associated with characterising isolates based solely on this characteristic. Grouping based on host-pathogen interaction is detected by the genetic background of the different cultivars available to distinguish strains (Correll, 1991). The assumption is that isolates with a shared host range and thus within the same *forma specialis*, are more similar genetically than isolates with other host specificities. The interpretation of this assumption is that isolates with a shared host range are likely derived from a single, particularly successful, pathogenic genotype (Kistler, 1997). However, this assumption does not conform to pathogenicity differences found among isolates of *C. dematium* on cowpea, as isolates even from the same field differed from non-pathogenic to highly pathogenic.

When the isolates of *C. dematium* were prepared for determining vegetative compatibility groupings, all the isolates that could form *nit* mutants were divided into four phenotypic classes (*nit*1, *nit*2, *nit*3 and NitM). The phenotypic classes obtained corresponded to those found in studies of *Fusarium* (Katan & Katan, 1988), *Neurospora* (Fin & Marzluf, 1987) and *Colletotrichum* (Brooker *et al.*, 1991). In the present study, *nit*3 mutants were predominantly produced, followed by *nit*1 and then NitM. Few isolates produced *nit*2 mutants. However, in studies of *Fusarium* species, *nit*1 and *nit*3 were produced in abundance and in almost equal numbers (Correll *et al.*, 1987; Elmer & Stephens, 1989). Although all *C. dematium* isolates were able to convert to *nit* mutants and were grouped according to phenotypic classes, it is not yet known whether phenotypically similar strains are genetically more related than dissimilar strains (Katan, 2000). In determining formation of heterokaryons among the *nit* mutants, some isolates were able to form heterokaryons and others did not. According to Brooker *et al.* (1991) and Benyon *et al.* (1995), formation of heterokaryons between some

Colletotrichum isolates but not others is a common characteristic of VCG studies on *Colletotrichum* species. Since the reproductive mode in many *Colletotrichum* species is mainly or exclusively vegetative and the only means of exchanging genetic material between two strains is via heterokaryosis (Katan, 2000), the isolates that could not form a viable heterokaryon with each other were considered to be genetically isolated.

Some isolates of *C. dematium* were weakly compatible with isolates from other VCGs, thus demonstrating cross-VCG compatibility and close relatedness between the VCGs. This phenomenon was observed by Correll *et al.* (1993) on *C. dematium* f.sp. *spinacia* causing anthracnose of spinach, and by Wasilwa *et al.* (1993) on *C. orbiculare* causing anthracnose of cucurbits. Vegetative compatibility groups are believed to represent clonal lineages within a species (Koenig *et al.*, 1997). For pathogenic isolates, VCGs are usually correlated with *formae speciales* (Appel & Gordon 1994). In cases of VCG incompatibility, it means horizontal transfer of deleterious cytoplasmic infection agents and viruses can be prevented. In an asexual fungus, such as *C. dematium*, this could also serve as a genetic isolation mechanism to prevent reassortment of genes that contribute to virulence (Anagostakis & Waggoner, 1981; Gobbi *et al.,* 1990). The low VCG diversity in *Colletotrichum* is correlated to asexual reproduction in these fungi since high VCG diversity reflects recombination of alleles at multiple *vic loci* and that indicates high genotypic diversity (Vaillancourt & Hanau 1994, Freeman *et al.,* 1998, Correll *et al.,* 2000). The low genotypic diversity in this study, as determined by RAMs -PCR analysis, compliments the low VCG diversity and thus conforms to these above factors.

When morphological characteristics, pathogenicity and VCG data were compared with RAMS analyses, *C. dematium* showed a high degree of variability as previously observed. Despite variations among the isolates, RAMS analysis consistently grouped together isolates from the same geographic area. There was no direct relationship between RAMS clusters and VCGs obtained. PCR

based detection of differentiation among isolates of *Colletotrichum* has been used widely (Mills *et al.*, 1992; Screenivasapradas *et al.*, 1996a; Neill *et al.*, 1997; Mesquita, 1998). Different isolates of different *Colletotrichum* species are shown to vary considerably with respect to pathogenicity and morphological traits from different hosts and within a host (Screenivasapradas, 1992; Kurame-Izioka *et al.*, 1997).

Although there is much variability among isolates of *C. dematium*, isolates from the same geographic area with similar morphological characteristics and pathogenicity were clustered together. RAMS analysis was able to determine genetic diversity of the *C. dematium* population. Therefore in *C. dematium*, on cowpea, pathogenicity of an isolate can be linked to its geographic area. The explanation could be that the cultivar used was resistant to isolates obtained from other geographic areas. The probability is that isolates may be specific to a particular cowpea cultivar, because only one cultivar was used in this investigation and isolates that were infectious in the field were non-pathogenic to the cultivar used. This implies that for a proper management of the disease, strains from one geographic area should not be introduced to other areas. Work is now needed to assess and compare the behaviour of a greater range of isolates of *C. dematium* from different cowpea varieties to investigate isolate-variety specificity.

3.6 Acknowledgements

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Table 3.1

Cultural and morphology characteristics of isolates of *Colletotrichum dematium* from *Vigna unguiculata* on potato dextrose agar and potato carrot agar

Group	Colony colour	Concentric Zonation	Sectoring	Conidia colour	Isolates
1	Pale mouse grey to mouse grey	Slight	None	Buff to salmon	Cd001, 2, 8 26, 32,33, 34, 35, 37, 38, 39, 40,41, 42, 43, 44, 45, 47, 48, 49, 50
2	Pale mouse grey to dark mouse grey	Prominent	Occasional	Salmon	Cd003, 4, 5, 6, 9, 10, 11, 14, 27, 28
3	Salmon to greyish sepia	Slight	None	Salmon to pale vinaceous grey	Cd019, Cd020
4	Pale mouse grey to mouse grey	Prominent	Occasional	Buff to salmon	Cd013
5	Pale mouse grey to olivaceous grey	Prominent	Occasional	Pale olivaceous grey	Cd012
6	Buff to greyish sepia	Prominent	None	Salmon	Cd021
7	Grey olivaceous to grey mouse	Prominent	None	Smoke grey to pale olivaceous grey	Cd022, Cd024
8	Saffron to ochreous	Prominent	Slight	Salmon to saffron	Cd031
9	Grey olivaceous to olivaceous black	Prominent	Occasional	Pale greenish grey to olivaceous gray	Cd023, Cd025
10	Smoke grey to olivaceous grey	Prominent	Occasional	Salmon to pale vinaceous	Cd007, Cd018
11	Pale olivaceous grey to dark olivaceous grey	Slight	None	Salmon to rosy buff	Cd016, Cd017
12	Olivaceous buff to grey olivaceous	Slight	Occasional	No visible conidia	Cd015

Table 3.2

Pathogenicity ranking of *C. dematium* isolates from different geographic areas

Isolate	Pathogenicity ranking[a]	Geographic area
Cd001	3	F1 GP
Cd002	3	F1 GP
Cd003	2	F1 GP
Cd004	2	F1 GP
Cd005	4	F1 GP
Cd006	4	F1 GP
Cd007	4	F1 GP
Cd008	4	F1 GP
Cd009	4	F1 GP
Cd010	4	F1 GP
Cd011	4	F1 GP
Cd012	4	F1 GP
Cd013	4	F2 GP
Cd014	2	F2 GP
Cd015	1	F2 GP
Cd016	4	F2 GP
Cd017	3	F2 GP
Cd018	1	ZIM
Cd019	1	ZIM
Cd020	2	ZIM
Cd021	3	ZIM
Cd022	4	ZIM
Cd023	4	KZN
Cd024	4	KZN
Cd025	4	KZN
Cd026	4	MZ
Cd027	4	MZ
Cd028	3	MZ
Cd031	2	F3 GP
Cd032	4	F3 GP
Cd033	4	F3 GP
Cd034	3	F3 GP
Cd035	4	F3 GP
Cd036	4	F3 GP
Cd037	4	F3 GP
Cd038	3	F3 GP
Cd039	3	F3 GP
Cd040	4	F3 GP
Cd041	4	F3 GP
Cd042	4	F3 GP
Cd043	4	F3 GP

Cd044	3	F3 GP
Cd045	4	F3 GP
Cd046	4	F3 GP
Cd047	4	F3 GP
Cd048	4	F3 GP
Cd049	4	F3 GP
Cd050	4	F3 GP

[a] Pathogenicity levels: 1-2 varies from non-pathogenic to slightly pathogenic and 3-4 were moderately pathogenic to highly pathogenic

Table 3.3

Comparison of isolates on the basis of clusters produced through RAMs analysis with pathogenicity on cowpea, vegetative compatibility groups and geographic area

Isolate No.	RAMs cluster[a]	VCG[b]	Geographic area[c]
Cd001	1	3	F1 GP
Cd002	1		F1 GP
Cd004	2		F1 GP
Cd006	3,4	1,3	F1 GP
Cd007	3,4	2	F1 GP
Cd005	2	1	F1 GP
Cd009	4		F1 GP
Cd010	4	1	F1 GP
Cd011	4	2	F1 GP
Cd012	4		F1 GP
Cd013	5		F2 GP
Cd016	5		F2 GP
Cd014	6	1	F2 GP
Cd015	6	1	F2 GP
Cd019	7	2,3	ZIM
Cd020	7	2	ZIM
Cd021	7		ZIM
Cd022	8	2	ZIM
Cd023	8		KZN
Cd033	9	1	F3 GP
Cd034	9	2	F3 GP
Cd035	9		F3 GP
Cd036	9		F3 GP
Cd037	9	1	F3 GP
Cd038	9	1	F3 GP
Cd039	9	1	F3 GP
Cd040	9	1	F3 GP
Cd041	9		F3 GP
Cd042	9	1	F3 GP
Cd043	9	1	F3 GP
Cd044	9	1	F3 GP
Cd045	9	1	F3 GP
Cd046	9	2	F3 GP
Cd047	9	1	F3 GP
Cd048	9	3	F3 GP
Cd049	9	1,3	F3 GP
Cd050	9	1,2,3	F3 GP

Cd026	10	1	MZ
Cd027	10		MZ
Cd028	10		MZ
Cd024	10	2	KZN
Cd025	10		KZN
Cd031	10		F3 GP
Cd032	10		F3 GP
Cd017	11		F2 GP
Cd018	11		ZIM
Cd003			F1 GP
Cd008		2	F1 GP

a Clustering according to RAMs analysis

b Vegetative compatible group

c GP = Gauteng Province (F1 = Flied 1, F2 = Field 2, F3 = Field 3); KZN = Kwazulu-Natal Province; Zim = Zimbabwe; MZ = Mozambique

Figure 3.1

Vegetative compatibility groups of *C. dematium* isolates based on growth patterns of complementary *nit* mutants. (A) Heavy wild type (VCG1), (B) Medium growth (VCG2) and (C) thin growth (VCG3)

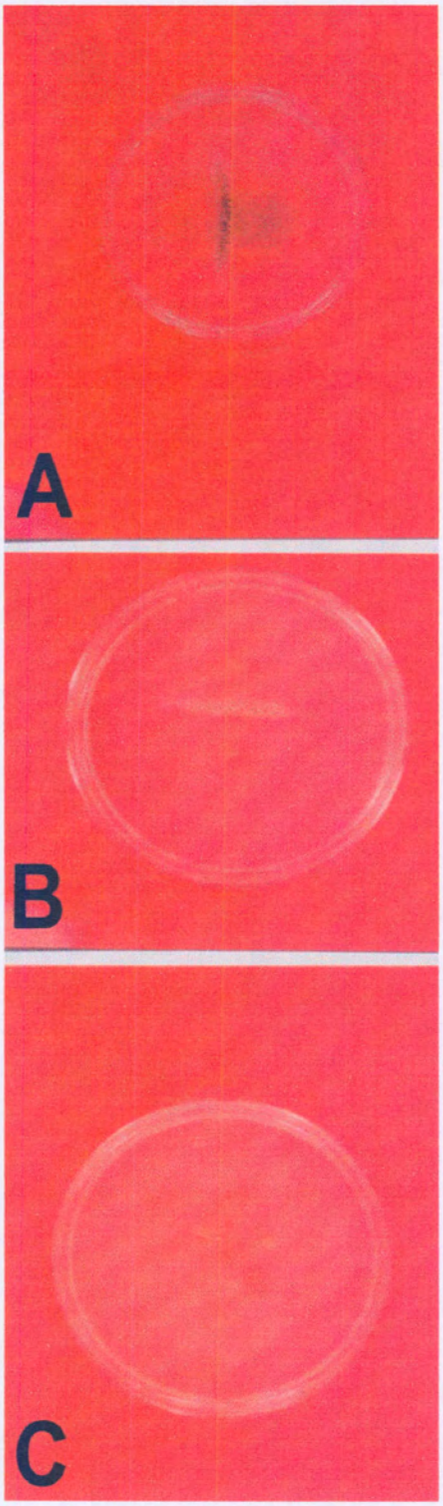
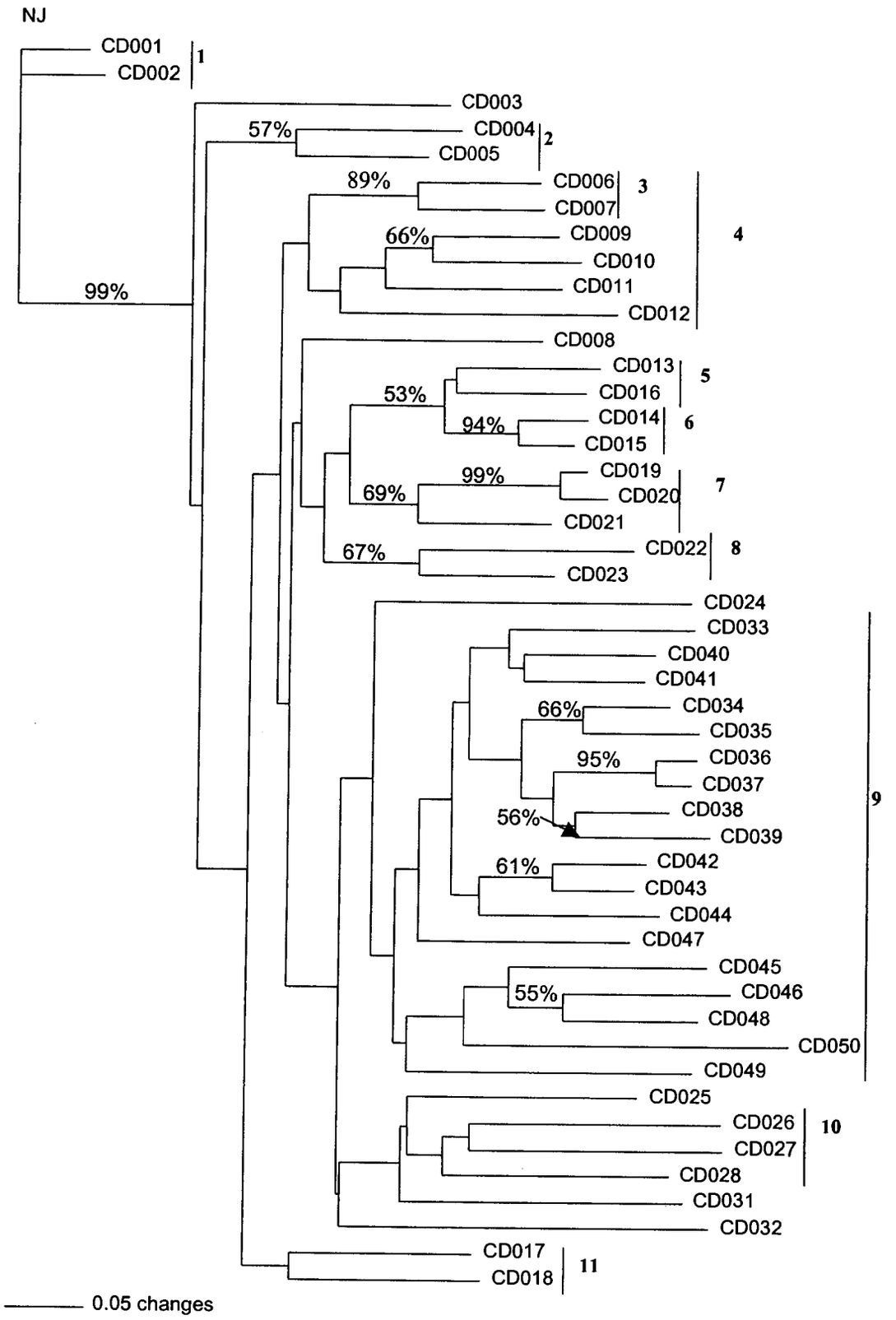

A
B
C

Figure 3.1

Vegetative compatibility groups of *C. dematium* isolates based on growth patterns of complementary *nit* mutants. (A) Heavy wild type (VCG1), (B) Medium growth (VCG2) and (C) thin growth (VCG3)

Figure 3.2

Neighbour-joining concensus tree produced from combined RAMs-PCR profiles to assess relatedness among 48 *C. dematium* isolates from cowpea



CHAPTER 4

THE NECROTROPHIC INFECTION STRATEGY OF *COLLETOTRICHUM DEMATIUM* ON *VIGNA UNGUICULATA*

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4.1 Abstract

The necrotrophic infection of cowpea by *Colletotrichum dematium* was studied on stems of a 6-week-old susceptible cowpea cultivar using a highly virulent isolate. Cowpea seedlings were inoculated with a conidial suspension of *C. dematium* and stem tissues were sampled at 6, 12, 24, 48, 52, 65, 72, 105 and 120 hours post inoculation (hpi). The samples were prepared for viewing with a light microscope, scanning, high resolution scanning and transmission electron microscopes. Necrotrophy at 48 hpi was characterized by subcuticular intramural colonisation of host tissue. Cell walls of cells near secondary hyphae were dissolving in advance of the hyphae, indicating presence of enzymes. However, the cuticle was not dissolved but pulled away from the dissolving cell wall. *C. dematium* exhibited destructive necrotrophic infection strategy starting at about 65 hpi. This was characterised by rapid destruction of host cells from the epidermis to the cortex. Acervuli formed once the parenchyma cells were completely dissolved and developed just beneath the cuticle. Pulvinate acervuli erupted through the cuticle at 72 hpi and released conidia. Conidia developed from the inner walls of conidiophores and two distinct shapes were observed. By 120 hpi, sporulation had ceased and acervulus contents had dissolved into a watery mass contained by the cuticle.

120 hpi, sporulation had ceased and acervulus contents had dissolved into a watery mass contained by the cuticle.

Keywords: acervulus, anthracnose, *Colletotrichum dematium*, cowpea, cuticle, destructive necrotrophy.

4.2 Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is a widely grown legume in tropical and subtropical Africa, cultivated for consumption of its leaves, pods and grain, thus providing a major source of protein for most rural people (Tindal 1983). The major constraint to cowpea cultivation is its susceptibility to numerous diseases and pests (Prasanna 1985). *Colletotrichum dematium* (Pers. (L) Grove) is one of the fungal pathogens causing anthracnose symptoms in cowpea (Lenne 1992). In South Africa, Smith & Aveling (1997) first observed *C. dematium* on cowpea in KwaZulu-Natal. They described the symptoms as tan brown to purple discolouration on the stem with sunken lesions containing black acervuli.

The initial stages of infection on the plant surface are the same for all *Colletotrichum* species and major differences become apparent after penetration (O'Connell *et al.* 2000). The infection processes vary and most *Colletotrichum* species exhibit different strategies of colonising the host tissue without provoking a hypersensitive reaction (Skipp *et al.* 1995). The most common is intracellular hemibiotrophy, which includes biotrophy when adhesion of spores, appressorium formation and intercellular proliferation of primary hyphae occurs (Skipp & Deveral 1972, O'Connell *et al.* 1985, Smith *et al.* 1999). This is usually followed by benign necrotrophy characterised by rupturing of cell membranes and cytoplasmic disorganisation, which indicates cell death (Bailey *et al.* 1992). In this case, a large proportion of cells may die before symptoms appear (Skipp *et al.* 1995). Contrary to benign necrotrophy, some *Colletotrichum* species follow destructive necrotrophy, which is characterised by abundant production of cell wall degrading enzymes capable of killing cells and releasing nutrients in tissue in advance of those containing hyphae (Bailey *et al.* 1992). The second strategy

subcuticular intramural colonisation. In this strategy, infection hyphae form mycelial networks in the periclinal and anticlinal walls causing swelling of cell walls. This suggests that fungal nutrition involves enzymatic degradation of wall material (Skipp *et al.* 1995).

The initial infection stage, the biotrophic phase of *C. dematium* on cowpea stems, was described by Smith *et al.* (1999). The periods of conidium germination, formation of appressoria, penetration of the host, formation of infection vesicles and inter- and intracellular ramification of primary and secondary hyphae were described. Acervuli were observed on the surface of the host at 70 h post inoculation (Smith *et al.* 1999). However, it is not known what type of necrotrophic infection strategy *C. dematium* exhibits. Hence, one of the aims of this paper was to study the infection of cowpea stems by *C. dematium* in order to characterise the necrotrophic phase.

In all *Colletotrichum* species, the length of time for initial infection tp occur varies, but eventually all of them revert to necrotrophy (Curry *et al.* 2002). The necrotrophic phase is characterised by the formation of acervuli, which involves massive sporulation on the surface of the necrosed host tissue (Onerisaron & Barker 1971, O'Connell *et al.* 1985, Parbey 1996). Two types of acervulus were described for *Colletotrichum* species by Sutton (1966). The pulvinate acervulus, which is described as the most common among *Colletotrichum* species, forms within the epidermal cell and depends on the pathogen to mechanically rapture the cell wall and tear the cuticle. The hypostromatic acervulus forms when setae and coniodiophores produced individually penetrate the cell wall and the cuticle (Sutton 1966). The second aim of this paper was to determine what type of acervulus *C. dematium* forms and to study the changes in the morphology of the acervulus and its contents, including conidium formation, during its development within a lesion.

4.3 Materials and Methods

4.3.1 Fungal culture

A culture of *C. dematium* isolated from cowpea was obtained from the National Collection of Fungi, Pretoria, South Africa (PPRI 6121). The culture was grown on potato dextrose agar (PDA) (Biolab, Halfway house, South Africa) at 25 ^{0}C under continuous UV-illumination.

4.3.2. Plant material

Cowpea seeds of a susceptible cultivar, Rhino, were planted in 15 cm diameter plastic pots containing pasteurised potting soil (4 seeds per pot) and maintained at approximately 25 ^{0}C in a greenhouse under natural light conditions. The plants were watered when required.

4.3.3 Inoculation

A spore suspension was prepared by pouring 10 ml of sterile distilled water over 7-day-old virulent cultures of *C. dematium* and agitating the Petri dishes (Smith *et al.* 1999). The stems of 6-week-old cowpea plants were painted to run-off with a spore suspension ($1x10^{5}$ conidia ml^{-1}) of *C. dematium* using a fine-thistle brush. Inoculated plants were placed in humidity chambers at 25 ^{0}C.

4.3.4 Scanning electron microscopy (SEM)

To study the host-pathogen interaction within the tissue, 4-5 stem sections per treatment, measuring 2-5 mm were excised at various time intervals (6, 12, 24, 48, 52, 65, 72, 105 and 120 h) after inoculation. Stem tissue and discs from cultures for conidial formation studies were fixed in phosphate-buffered glutaraldehyde (2.5%) for 1.5 h, then rinsed in the same buffer three times for 10 min each. Material was post-fixed in 0.25% w/v aqueous osmium tetroxide (OsO_4) for 1 h, followed by three successive rinses in distilled water. The material was dehydrated in a graded ethanol series and critical point dried in a Bio-rad critical point dryer. Dehydrated material was mounted on stubs and coated with

gold in a Polaron E5200 sputter coater (Watford, England) and examined with a JEOL JSM 840 scanning electron microscope operated at 5 kV. Samples used for studying the changes in the cuticle were made conductive by exposing them to RuO_4 vapour for 1 h (Peacock *et al.* 1998) and viewed with a JEOL 6000F in-lens field emission scanning electron microscope.

4.3.5 Freeze fracturing

Stem samples of 1 cm in length were cut from the inoculated stem tissue 72 hours post inoculation (hpi). Samples were fixed in 0.5% w/v OsO_4 in 0.075 M phosphate buffer for 2.5 h at room temperature. The samples were prepared using the "osmium-tannic acid-osmium method" of Tanaka & Mitsushima (1983). The fixed samples were rinsed with buffer and treated with 15, 30 and 50% dimethyl sulphoxide (DMSO) for 30 min in each solution. The samples were frozen in liquid nitrogen and either fractured with a scalpel and polished with 240-grid emery paper. The split pieces were thawed in 50% DMSO, rehydrated in 30 and 15% DMSO for 15 min in each solution and rinsed with buffer solution until all DMSO was removed. Samples were then transferred to buffered 0.1% OsO_4 for 72 h at 4 ^{0}C. The osmium solution was replenished at 24 and 48 h. The samples were post-fixed in 1% OsO_4 for 1 h, treated with 2% tannic acid overnight followed by 1% OsO_4 for 1 h. Samples were dehydrated in a graded ethanol series and critical point dried. Dried samples were fixed on stubs with carbon adhesive tabs and conductive carbon cement and coated with chromium in an ion-beam coater with a rotating specimen holder. The metal-coated samples were examined using a JEOL JSM 840 scanning electron microscope operated at 5 kV.

4.3.6 Transmission electron microscopy (TEM)

Stem tissue and culture discs measuring 1-2 mm in length were prepared for TEM. Samples were fixed as described for SEM. The fixed samples were dehydrated in a graded ethanol series followed by infiltration with 1:1 epoxy resin: ethanol for 1 h and then 4 h in 100% resin. Infiltrated material was embedded in 100% epoxy resin and polymerised in an oven at 60 ^{0}C for 48 h. Sections were cut using a Reichert Jung Ultracut E microtome with a diamond knife, contrasted with 4% aqueous uranyl acetate for 15 min and lead citrate for 2 min. Monitor sections were examined with a Nikon light microscope. Sections were also examined with a Philips EM301 transmission electron microscope operated at 60 kV.

4.4 Results

The ontogeny of the infection process of *C. dematium* on cowpea stems from 9 - to 48 hpi confirmed findings by Smith *et al.* (1999). Cell distress was apparent at 48 hpi where the cell walls of cells close to the invading hyphae (Fig. 4.1a) were dissolved. Four hours later, on material polished with emery paper and viewed with a high-resolution scanning electron microscope (HRSEM), intracellular hyphae were spotted in the cortex (Fig. 4.1b). As the incubation period progressed to 65 hpi, material showed marked change when viewed with the light microscope. There was massive proliferation of secondary hyphae throughout the entire tissue, with the epidermis and endodermis completely destroyed and the only cells still recognisable were the collenchyma (Fig(s). 4.1c & 4.1d). Hyphae proliferated both inter- and intracellularly and between the primary and secondary cell walls of sclerenchyma cells (Fig. 4.1d). During this stage, the cell walls of sclerenchyma cells retained their shape but the plasmalemma pulled away from the cell wall.

Comparison of the cuticle of uninfected material (Fig. 4.2a) with that of infected material (Fig. 4.2b), showed that the cuticle pulled away from the cell wall in close proximity of the invading hypha. This was further highlighted by TEM (Fig.

4.2c) where the cuticle seemed to pull away in advance of the hypha, while the cell wall was dissolving close to the hypha.

Lesion formation at 72 hpi began with the accumulation of conidiogenous cells (stroma) beneath the cuticle. When uninfected material (Fig. 4.3a) and infected host material were freeze fractured and polished with emery paper, visualisation of different tissue types and internal bound organelles was possible. A fracture through the lesion of host material incubated for 72 h (Fig. 4.3b) shows complete destruction of cellular structures from the epidermis inward towards the cortex. Higher magnification of the lesion (Fig. 4.3c) shows an intact cuticle while the epidermis and underlying cells were completely destroyed. Aggregated conidiogenous cells were clearly visible thus depicting the onset of the formation of an acervulus. A TEM section through a forming lesion showed that differentiating conidiogenous cells were characterised by electron translucent lipid bodies, as well as septa, distinct nuclei and crystal-like clusters situated near the nuclei (Fig. 4.3d).

On culture plates, conidiogenous cells were visible after 36 h, with swollen tips formed as side branches of mycelia (Fig. 4.4a). An emerging conidium (Fig. 4.4b) appeared obovoid in shape. As the conidium enlarged, the shape changed to clavate with a smooth rounded head (Fig. 4.4c). Conidium formation on host material was similar to that observed in culture. TEM revealed emerging obovoid conidium from infected plant tissue (Fig. 4.5a) and other conidia had a pointed end (Fig 4.5b). Detached conidia were surrounded by copious amounts of extracellular matrix (Fig. 4.5c). This matrix was visible from within the inner layer of the conidiophores and was continuous around the released conidia (Fig. 4.5c). Two distinct shapes of conidia were observed. One was slightly clavate in shape with a smooth rounded head and a slightly pinched distal end (Fig. 4.5d). The other characteristic was boat-shaped (fusiform), with both ends tapering and pointed (Fig 4.5e).

contained by the cuticle (Fig. 4.6a). The contents of the sporulating acervulus such as setae and conidiogenous cells were still visible at 105 hpi. At 120 hpi, cellular contents of the conidiophores disintegrated and the contents of the acervulus could no longer be distinguished (Fig. 4.6b). During this stage only the cuticle that contained the necrosed contents of the stem remained. Termination of conidium formation was characterised by collapsed conidiophores and disrupted hyphae, most of which lacked cellular content (Fig. 4.6c).

4.5 Discussion

Colletotrichum dematium on cowpea exhibited a prolonged biotrophic phase of more than 40 h, before necrotrophy commenced at approximately 48 hpi. A prolonged biotrophic phase is characteristic of some *Colletotrichum* species such as *C. lindemuthianum* on bean (O'Connell & Bailey 1986), *C. gloeosporioides* on maize (Churchill *et al.* 1988), *C. destructivum* on cowpea (Latunde-Dada *et al.* 1996) and *C. truncatum* on lentil (Chongo *et al.* 2002). In these cases the fungus invades the host without killing the cells thus avoiding triggering a hypersensitive reaction (Perfect *et al.* 1999). The first phase of necrotrophy in *C. dematium* was characterised by rapid ramification of hyphae both inter- and intracellularly with hyphae found in the periclinal walls of collenchyma cells. This suggests that *C. dematium* exhibits subcuticular intramural colonisation, which is usually common in *Colletotrichum* species that cause fruit rot (Bailey *et al.* 1992). Immediately after that unrestricted rapid destruction of the cowpea stem tissue followed. This stage was characterised by dissolution of cell walls of cells near colonising hyphae, indicating possible secretion of enzymes. This is similar to the necrotrophic phase of *C. lindemuthianum* on bean where the host cell wall degraded up to 200 μm in advance of infection (O'Connell *et al.* 1985). Production of enzymes during infection by *Colletotrichum* species (Wijesundera *et al.* 1989, Wharton *et al.* 2001) is also associated with appearance of lesions on the host surface of *Phaseolus vulgaris* L. infected by *C. lindemuthianum* (Bailey *et al.* 1992).

the host surface of *Phaseolus vulgaris* L. infected by *C. lindemuthianum* (Bailey *et al.* 1992).

Lesions on the cowpea stem were contained within the cuticle and were characterised by aggregation of conidiogenous cells at 72 hpi. The lesions were water-soaked and gradually spreading from site of initial infection to uninfected stem tissue. Spreading lesions are associated with dissolution of cell walls and killing of cells in advance of infected cells (O'Connell & Bailey 1988). However, in some *Colletotrichum* infection strategies, lesions are not as a result of destructive necrotrophy (Skipp *et al.* 1995), as in our study, but due to a hypersensitive reaction to contain the fungus before it spreads (O'Connell *et al.* 1985).

Acervuli were formed over the water-soaked lesions. At this stage the cuticle was still intact, containing the acervulus and its contents. In all *Colletotrichum* species, an intact cuticle is required for the production of acervuli (Sutton 1966). In this study the acervuli formed in the epidermis beneath the cuticle where conidiogenous cells collected and differentiated to form conidiophores and setae. The type of acervuli observed in *C. dematium* was pulvinate, where the expanding conidiogenous cells ruptured the cuticle, as observed by Smith *et al.* (1999). The pulvinate acervuli were formed over dissolved cellular mass. In contrast, the acervuli of *C. fragaria* and *C. acutatum* were formed over slightly disrupted epidermal and subepidermal cells (Curry *et al.* 2002).

Formation of conidia in *Colletotrichum* species is described as phiallidic, that is, the first conidium is formed holoblastically and the rest follow in a basipetal succession (Baxter *et al.* 1983). It was clear in this study that the conidium that is formed holoblastically has a smooth frontal end and a pointed distal end. Since only one conidium is formed holoblastically, this shape is rarely found in conidium masses. The fusiform shape that is characteristic of *C. dematium* is the shape of all the other conidia that are formed in a basipetal manner. Termination of conidiogenesis was characterised by dissolution of acervulus contents into a watery-mass, all contained by the cuticle.

The destructive necrotrophic phase was characterised by cell death, formation of water-soaked lesions and production of numerous acervuli. These infection strategies make C. *dematium* an effective anthracnose fungus and thus the pathogen poses a serious threat to cowpea production.

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Figure 4.1

Fig. 1a Light micrograph of cowpea stem material at 48 hpi showing initiation of the necrotrophic phase of *C. dematium* in parenchyma cells, cells near the invading hyphae were disrupted (arrows) (Bar = 1 µm). Fig. 1b HRSEM of hyphae (hy) of *C. dematium* growing intracellulary in the cortex cells of freeze-fractured material of cowpea stem at 52 hpi (Bar = 1 µm). Fig. 1c Heavily infected material of cowpea stem at 65 hpi, sclerenchyma cells (sc) (Bar = 1 µm). Fig. 2d Higher magnification of secondary hyphae (hy) of *C. dematium* within collenchyma cells and between primary and secondary cell walls (Cw) (Bar =1 µm).

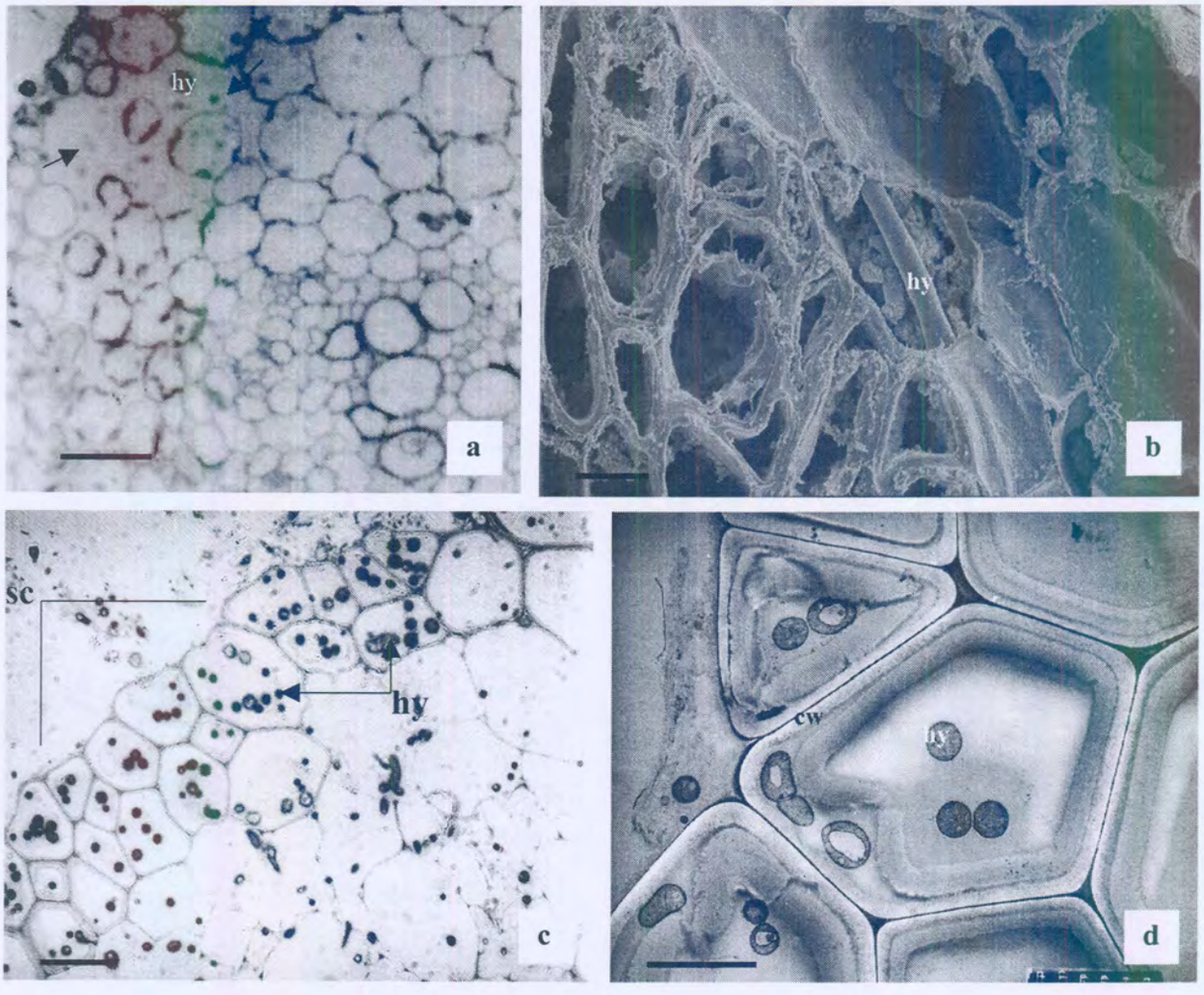


Figure 4.2

Fig. 2a HRSEM of an intact cuticle (Cu) and cell wall (Cw) of uninfected cowpea stem (Bar = 1 µm). Fig. 2b Cuticle (Cu) separated from the cell wall (Cw) of cowpea stem in the vicinity of a hypha (hy) of *C. dematium* (Bar = 1 µm). Fig. 2c TEM of the separation of cuticle (Cu) from cell wall (Cw) in close proximity to a hypha (hy) of *C. dematium*. Asterix shows a torn part of the section where epoxy-resin separated from the cuticle (Bar = 1 µm).

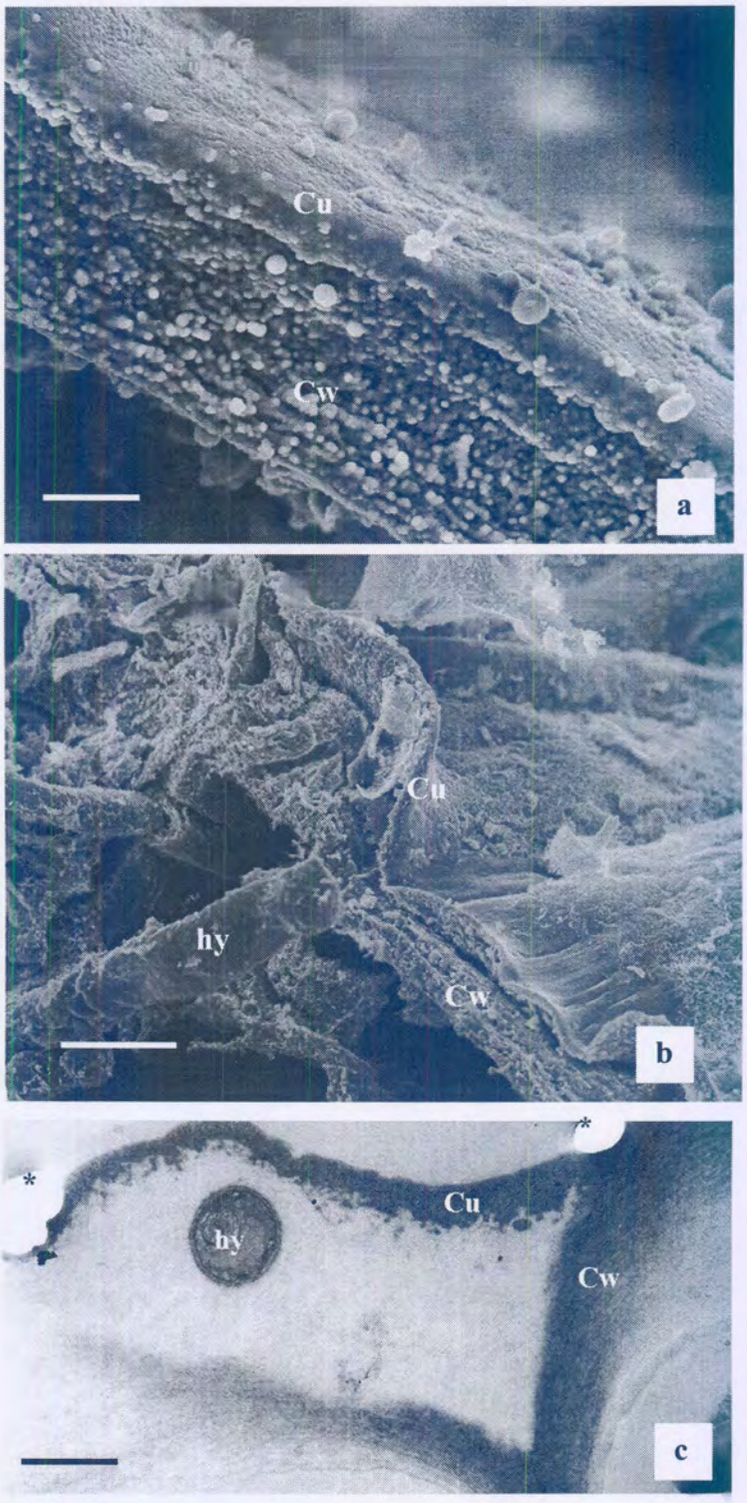


Figure 4.3

Fig. 3a Freeze-fractured cowpea stem material of a cross-section of uninfected tissue (Bar =100 µm). Fig. 3b A lesion (L) formed 72 hpi by *C. dematium* on a cowpea stem, spreading from the cuticle (Cu) towards the pith (P) (Bar = 100 µm). Fig. 3c. Higher magnification of the lesion showing differentiated conidiogenous cells (cc) marking the initial stage of conidiogenesis beneath the cuticle (Cu) (Bar = 10 µm). Fig. 3d a cross section of *C. dematium* grown in culture at 36 h shows conidiogenous cells characterised by crystal-like structures (cr). On the right side of the micrograph, elongated conidiophores with distinct nuclei (n) and septa (s) are visible (Bar = 1 µm).

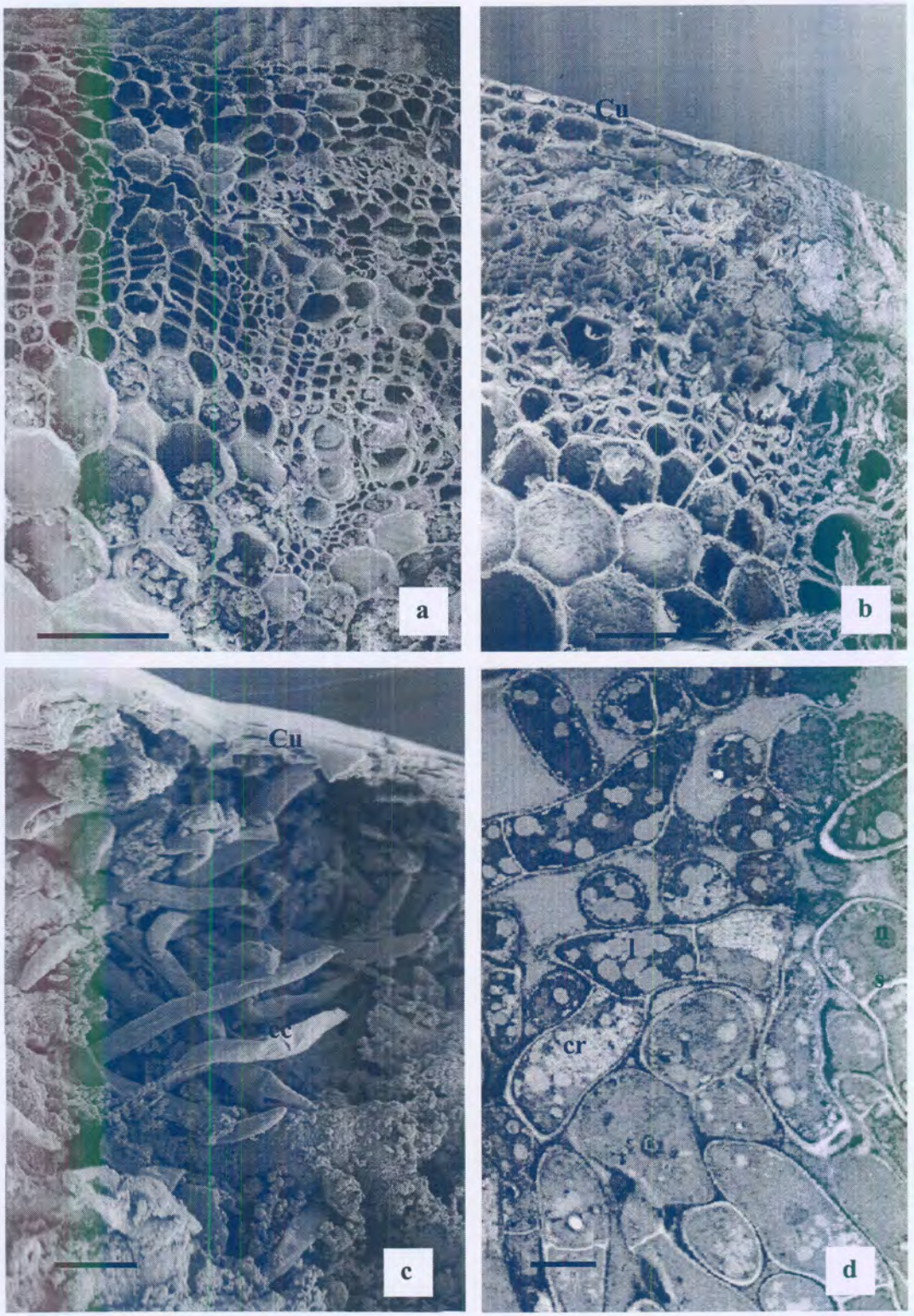
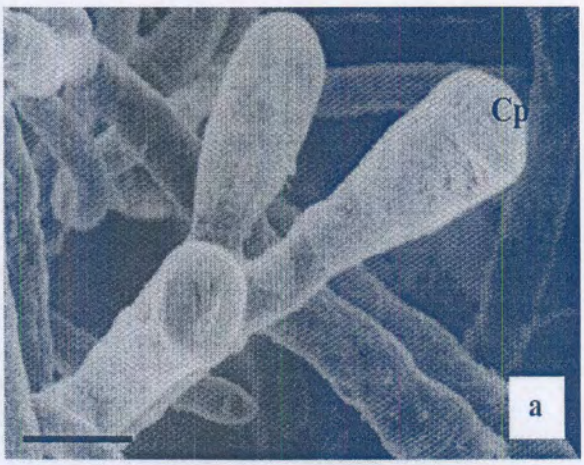
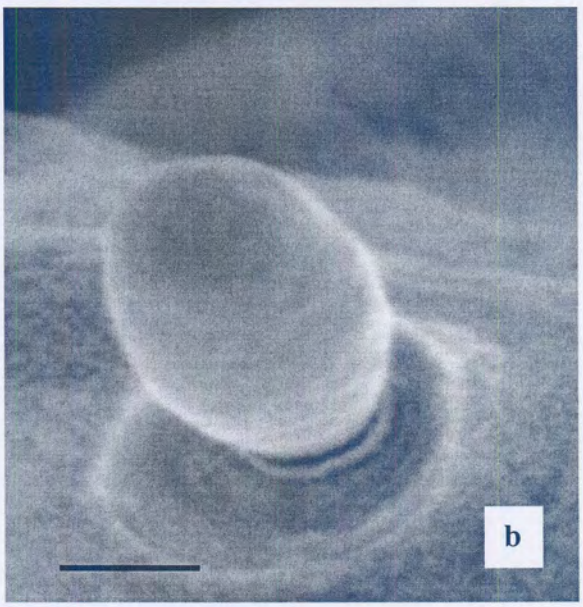


Figure 4.4

Fig. 4a Young conidiophores (Cp) of *C. dematium* characterised by swollen tips before formation of conidia (Bar = 1 µm). Fig. 4b Initiation of conidiogenesis at 60 hpi viewed with SEM showed an emerging conidium (Bar = 1 µm). Fig. 4c The obovoid shape of a developing conidium of *C. dematium* still attached to the conidiophore (Cp)(Bar = 1 µm).





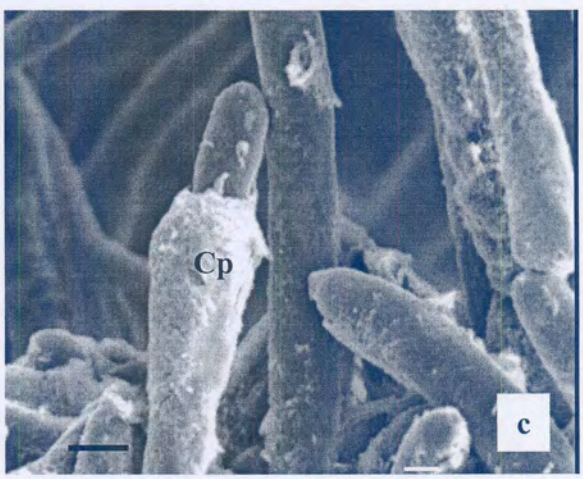


Figure 4.5

Figs. 5a & 5b Both TEM micrographs show conidia still in different stages of development attached to conidiophores. Fig 5c A cellular matrix (ecm) surrounds detached mature conidia of *C. dematium* (Bar =1 µm). Fig. 5d A mature clavate first conidium of *C. dematium*, characterised by a smooth head end and pinched or slightly pointed distal end (Bar = 1 µm). Fig. 5e A mature fusiform shaped conidium is characteristic of *C. dematium* (Bar = 1 µm).

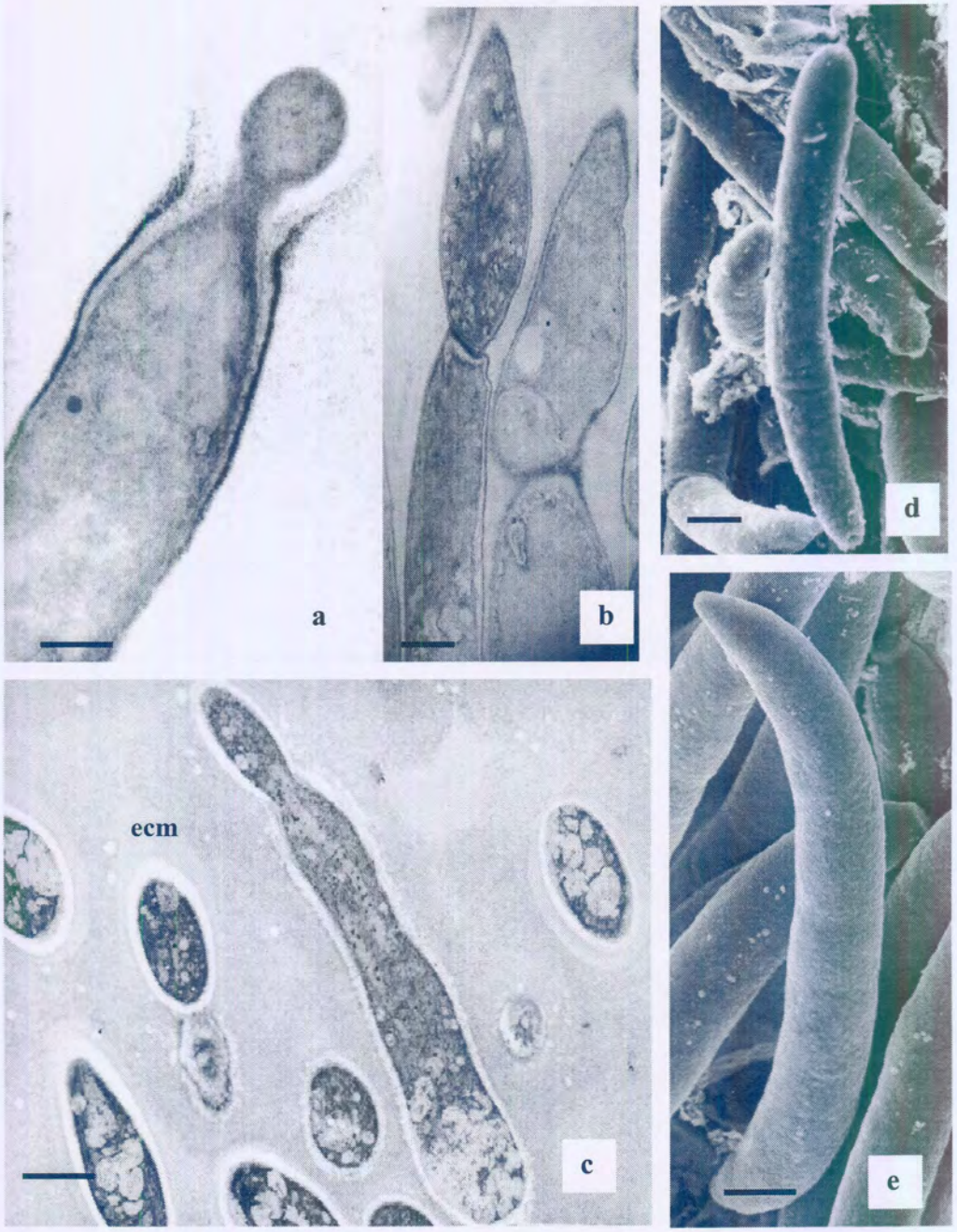
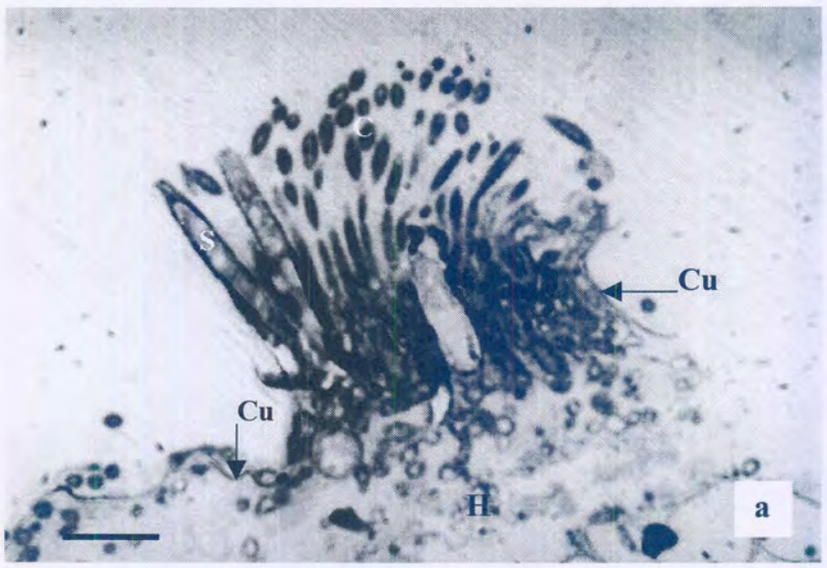
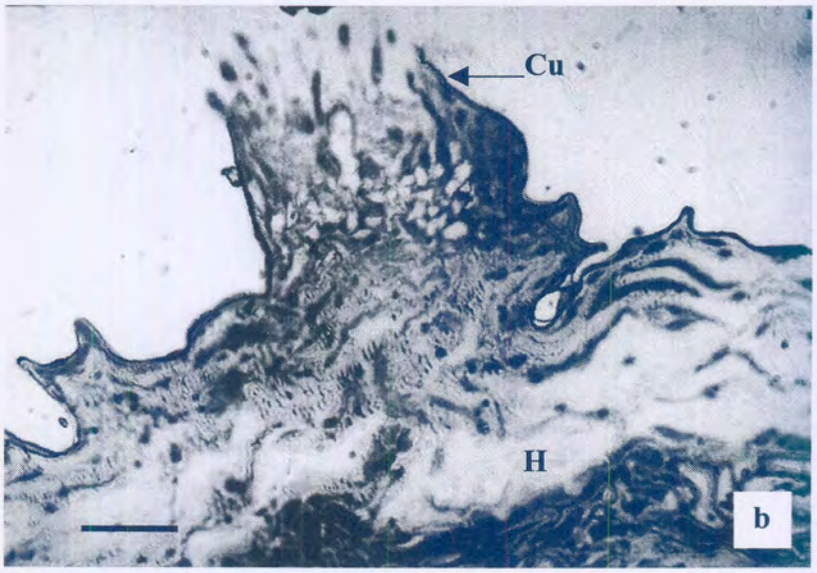
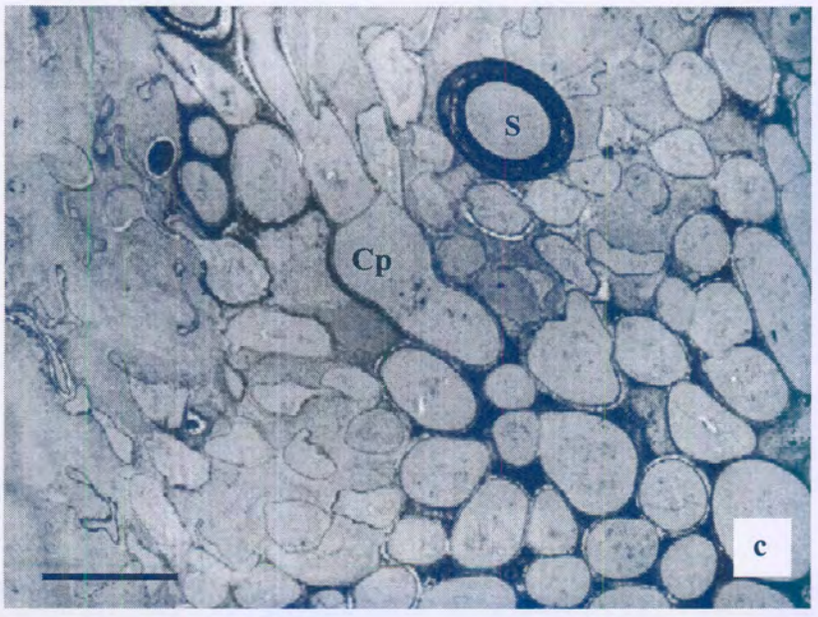


Figure 4.6

Fig. 6a An actively sporulating acervulus of *C. dematium* on cowpea stem, 72 hpi. The contents contained within the cuticle (Cu), setae (S) and conidia (C) of the acervulus are visible although there is no cellular differentiation within the host tissue (H) (Bar = 7 µm). Fig. 6b A mature acervulus after completion of conidiogenesis 120 hpi, the ruptured cuticle (Cu) encloses the acervulus while cellular differentiation of host tissue below the acervulusis non-existent (Bar = 7 µm). Fig. 6c Termination of conidiogenesis by *C. dematium* is characterised by collapsed conidiophores (Cp) and disrupted hyphae most of which lack cellular content, and setae (S) (Bar = 5 µm).







CHAPTER 5

PATTERNS OF POLYPHENOLIC COMPOUNDS IN COWPEA CULTIVARS RESISTANT AND SUSCEPTIBLE TO *COLLETOTRICHUM DEMATIUM*

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5.1 Abstract

Four cowpea (*Vigna unguiculata*) cultivars differing in seed coat colour - cream with black eye (CH84), reddish brown (PAN311), brown (Encore) and mottled brown and cream (Rhino) were analysed using a combination of histochemical, biochemical and microscopy techniques. High performance liquid chromatography indicated that kaempferol was the main flavonoid detected in the four cultivars but quercetin was not detected. Seed coats of brown seeds contained a higher concentration of soluble phenolics. These seed coats also emitted a strong fluorescence at 365 nm showing the location and concentration of catechin, tannins and flavonoids. The crude extracts of the brown cultivars prevented germination of conidia of *C. dematium* at 0.1 g ml^{-1}. It was observed that brown cowpea seeds were more resistant to *C. dematium*.

Keywords:, flavonoids, fungal toxicity, kaempferol, polyphenolics, seed coat .

5.2 Introduction

Cowpea (*Vigna unguiculata* [L.] Walp)., known also as southern pea or blackeye pea, is one of the worlds most important food crop legumes (Konowicz *et al.* 1997). It is planted primarily for its edible seeds and the young leaves are harvested and consumed as a vegetable (Nielsen *et al.* 1997). Cowpea seeds

exhibit a wide range of colours. Knowledge of the genetics of seed coat colour in cowpea is due mainly to the work of Spillman (1912), Harland (1920) and Spillman & Sando (1930). Phenols and their derivatives, mainly tannins, are responsible for the brown and black colours, while the bright colours are mainly due to flavonoids and anthocyanin (Werker 1997). The pigmentation of the seed may be located in various tissues such as the embryo, endosperm and seed coat. However, the seed coat is most often the determinant of seed colour (Werker 1997). The principal pigments in cowpea seeds are glycosides of cyanidin with some delphinidin and malvadin (Nozzolillo & McNeill 1985). Flavonoid compounds that are always present in cowpeas are mainly aglycones of kaempferol, quercetin and isorhamnetin (Lattanzio *et al.* 1997).

The cowpea plant is susceptible to a wide range of diseases and pests (Williams 1975). Recently, *Colletotrichum dematium* (Pers. (L) Grove) was found to cause severe anthracnose on cowpea in South Africa (Smith & Aveling 1997). There are no studies relating the colour of cowpea varieties to resistance or susceptibility to *C. dematium*. However, factors such as the rate of imbibition (Powell & Matthews 1978; Powell 1989), and germination and ageing (Abdullah *et al.* 1991) have been related to pigmentation in cowpea seeds (Morrison *et al.* 1995). A study by Legesse & Powell (1992) also revealed that cream/beige cowpea cultivars showed evidence of imbibition damage, poor vital staining with tetrazolium and a high rate of solute intake, as compared to coloured seeds with a slow imbibition rate. In other plant species, the colour of the testa has been related to its tannin content (Cabreba & Martin 1989). In relation to seed colour and fungal diseases, Stazs *et al.* (1980) discovered that coloured pea seed coats were not penetrated by *Pythium ultimum* Trow 100 hours after planting, whereas, uncoloured seeds were penetrated within 40 hours. However, in a study on the resistance of cowpea to *Macrophomina* and *Xanthomonas* and its association with seed colour, it was discovered that seed colour had no influence on resistance (Higuera & Murty 1987). In previous experiments, it was found that

dark coloured cowpea seeds tended to be more resistant to fungal attack and storage fungi as compared to white or cream coloured seeds (Kritzinger 2000).

The present work was undertaken to establish the patterns of polyphenolic compounds in differently coloured cowpea seeds, and to investigate if the seed coat colour has an influence on the resistance of the cowpea to *C. dematium*.

5.3 Materials and Methods

5.3.1 Plant material

Four cowpea cultivars (cv.) were obtained from the Agricultural Research Council- Small Grain Crops Institute, Potchefstroom, South Africa. These were selected for resistance or susceptibility to a virulent strain of *C. dematium* (PPRI 6121, obtained from the National Collection of Fungi, Pretoria, South Africa) based on a prior experiment (Pakela & Aveling, internal report). The descriptions of the cowpea seeds are listed in Table 5.1.

5.3.2 Preparation of samples

One hundred seeds per genotype were soaked in cold water for 4 h and seed coats were manually removed. The seed coats were frozen at -70^{0}C for 1 h, freeze-dried for 24 h and ground into a fine powder using a mortar and pestle.

5.3.3 Methanol extraction

A sample of the seed coat powder measuring 0.7 g was weighed and 1 ml 50% methanol was added. The material was vortexed for 1 min, shaken for 30 min, centrifuged for 5 min and the supernatant was collected. The seedcoat powder was extracted twice more. The supernatant was concentrated by freezing at -70^{0}C and freeze-drying overnight, then weighed and re-suspended in 50% methanol.

5.3.4 Boiled water extraction

The seed coats (0.7g ml^{-1}) were immersed in boiling water for 1 h. The mixture was then vortexed, centrifuged for 10 min and the supernatant was collected. This procedure was repeated twice more. The supernatant was concentrated by freezing at -70^{0}C and freeze-drying overnight, then weighed and re-suspended in sterile distilled water.

5.3.5 Determination of total soluble phenolics

For determining total soluble phenolics, 170 µl distilled water and 5 µl of the seed coat extract were mixed into each well of an ELISA plate (Emerschem) followed by 50 µl of 20% sodium carbonate. Folin-Ciocalteu's reagent (25 µl) (SIGMA) was added as a colourimetric indicator. A blank, consisting of identical composition but replacing the sample with water, served as a control. ELISA plates were incubated at 40^{0}C for 30 min. The experiment was repeated three times for each cultivar. The absorbency was measured at 690 nm with a Multiskan Ascent V1.24 Version 1.3.1. Total soluble phenolics were expressed as an equivalent of milligrams of gallic acid (standard) per gram of dry weight (dw), i.e., [(Absorbency - blank) + gallic acid standard (0.0109)] / dw. Difference between means was compared using Fisher's Protected least significant difference test.

5.3.6 High performance liquid chromatography (HPLC)

The seed coat extracts prepared above were filter-sterilised through a 0.22 µm filter. The standards, kaempferol and quercetin (Sigma-Uldrich, Germany) were prepared as 1.0 mg ml^{-1} and then diluted to 0.5 mg ml^{-1}. Standard solutions were injected immediately before the seed coat extracts. Twenty microlitres of the extracts were injected. A C_{18} column was used. For quantification, a solvent system (ethanol: water) was used at a flow rate of 1.0 ml min^{-1} and detection was at 200 to 600 nm (extraction wavelength was 280 nm). For qualitative analysis, a gradient method used: 9 ethanol: 81water to 9 water: 81 ethanol over 10 min with a total analysis time of 20 min. The results were analysed using a 1050 Hewlett

Packard HPLC system, equipped with a computer-aided spectrophotometric photodiode array detector 1040 Hewlett Packard.

5.3.7 Anti-fungal activity of cowpea seeds

The seed coat extracts were filtered and concentrated to dryness in an oven at 50^0C. After determining the weight of the extract, a stock solution consisting of 0.5 g was suspended in 5 ml sterile distilled water to form a 10% stock solution. For the kaempferol and quercetin standards 0.1 g was weighed and suspended in 1 ml of sterile distilled water. Dilutions from 90% to 20% of the stock solution were made. A volume of 160 µl of the diluted extract mixtures were mixed with 10 µl of a *C. dematium* conidium suspension ($1x10^5$ conidia ml^{-1}) in each well of an ELISA plate (Emerschem). Plates were incubated at 25^0C for 6, 12 and 24 h and then examined for germination of conidia and hypha formation. Complete inhibition of conidium germination was required for the extract to be declared bioactive. Controls were likewise prepared containing sterile distilled water. A volume of 2 µl of lactophenol in cotton blue was added to each treatment. The mixture was placed on a glass side and viewed with a light microscope. Conidium germination was determined by direct counting of 25 germinated conidia per treatment. Each treatment was repeated three times.

5.3.8 Histochemistry

Seeds were soaked in sterile distilled water and manually sectioned. For detection of flavonoid compounds, 2-aminoethyldiphenyl borinate (Neu's reagent) was used. The sections were immersed in the reagent for 1-2 min and then mounted in 15% glycerine water. Sections were also immersed for 15 min in citric acid: boric acid (5:5 w/v) in 100 ml absolute ethanol (Wilson's reagent). The sections were viewed with a fluorescent microscope. Proanthocyanins give blue or green violet spots and flavonoids give yellow, orange, red or green fluorescent spots (Dai *et al.* 1996). To locate catechins and condensed tannins, sections were stained with vanillin-HCl and viewed with a Nikon light microscope at 40X.

5.4 Results

5.4.1 Total soluble phenolics

There were significant differences in total soluble phenolic compositions obtained among the different seed types. In general, there were no significant differences in the amounts of total soluble phenolic compounds obtained from seed coats extracted with methanol and with water (Figure 5.1). Encore had a significantly higher phenolic content in methanol extracts and there was no significant difference amongst other cultivars. Extraction with boiling water indicated the concentration of total soluble phenolics was significantly higher on cv. PAN311 (Table 5.2).

5.4.2 High performance liquid chromatography

Analytical HPLC traces showed that flavonoid profiles of these four genotypes were similar (Figure 5.2). Four different compounds were eluted (A, B, C, D) from the cowpea seed coat extracts. Coinjection of the standards with the extracts increased the area and height of the peaks (similar to peaks of the standards when injected alone) and confirmed their identification. Peak A in all the extracts was confirmed as kaempferol. Quercetin was not detected in any of the extracts. There were no significant differences in the concentrations (peak heights) of all the compounds eluted from the cultivars extracted with methanol. In these extracts, three compounds were detected in Rhino, CH84 and Encore and only PAN311 had a fourth minor compound. In extracts with water, compound B was significantly higher in CH84.

5.4.3 Histochemistry

Chemical reactions with vanillin-HCl viewed with light microscopy showed that tannins in dark brown seeds were evenly distributed as a thick, darkly pigmented layer on seed coats. The seed coat of CH84 (cream coloured with a brown eye) was stained a light red (Figure 5.3a). The stain was uniformly distributed in the seed coat while the cells of the endosperm were unstained. In the mottled brown and cream seed coat of cv. Rhino, light and dark red spots were visible, depicting

the cream and brown colours of the seed coat (Figure 5.3b). In the reddish brown line, Encore, the seed coat was stained a dark red and there was a darker red line in the centre of the seed coat (Figure 5.3c). The endosperm cells of these cultivars were stained red. Cultivar PAN311 showed an intensely pigmented seed coat with a blackish-red thick line in the centre (Figure 5.3d).

Detection of flavonoids with Neu's or Wilson's reagents indicated that dark brown seeds emitted a strong blue fluorescence during excitation with ultra-violet light at 365 nm. When these were observed with a blue light (420 nm), bright green spots were visible in some areas. The fluorescence was less intense when cv. CH84 (Figure 5.4a) was excited with blue light and there was no fluorescence under UV light. The brown and cream cv. Rhino showed yellow spots clustered together with dark spots in between. The yellow spots indicated the presence of flavonoids in the brown areas of the seed coat (Figure 5.4b). Encore showed green lines on the seed coat indicating the location of proanthocyanins and flavonoids (Figure 5.4c). Cultivar PAN311 showed intense fluorescence throughout the seed coat (Figure 5.4d).

5.4.4 Anti-fungal activity of cowpea seeds

High concentrations of the crude extracts of all the cultivars showed significant fungitoxic activity to conidial germination. After 12 h conidium germination was detected in concentrations of the crude extract as well as the flavonoid standards diluted from 60 to 20%. At 24 h, the kaempferol extract was toxic to *C. dematium* conidia from 100 to 80% dilution, Encore and PAN311 were fungitoxic from 100 to 70% and Rhino from 100 to 90%. Quercetin and cultivar CH84 were fungitoxic only at 100% concentration of the crude extract (Table 5.3).

5.5 Discussion

Cowpea varieties exhibit a wide range of seed coat colours, from white, cream, different shades of brown and red, to black (Singh & Allen 1985). The data indicate that seedcoat colour has been linked to the resistance of the cultivar to the cowpea pathogen *C. dematium.* Brown seeds, which were resistant to *C. dematium*, exhibited a strong fluorescence of catechins, tannins and flavonoids. This is the first study where the colour of the cowpea seedcoat has been correlated to resistance to a fungal pathogen. In previous studies, the colour of the cowpea seed has be linked to storage pests and fungi, whereby, cream coloured seeds were more susceptible to storage pests than dark coloured seeds (Kritzinger 2000). White cowpea seeds were also found to be prone to imbibition damage resulting in low vigour of the seeds (Legesse & Powell 1992). The dark brown cultivars Encore and PAN311, contained a higher concentration of soluble phenolic compounds than the cream coloured cultivar CH84. The seedcoat colour of dark and brown seeds is mainly due to tannins while the bright colours are mainly due to flavonoids and anthocyanins (Werker 1997). Tannins in seeds are a form of defence against herbivores, they react with proteins to form tannin complexes resulting in indigestibility of the seed (Cabrebra & Martin 1989).

Kaempferol was the only flavonoid detected in all the cowpea cultivars tested. This confirms previous work by Lattanzio *et al.* (1997). In their study, kaempferol and quercetin were found to be major flavonoids in the leaves of the cultivated cowpea lines. Kaempferol has been detected as a major flavonoid in bean genotypes of different colours , while it is found only in spotted seeds of *Vicia faba* L. (Nozzolillo *et al.* 1998). Although quercetin was not detected in our cultivars, it is found mainly in the white seedcoats of *V. faba* (Nozzolillo *et al.* 1998). Flavonoid constituents in cowpea have been used mainly to assess inter and intraspecific relationships among wild and cultivated species (Lattanzio *et al.* 1997).

The fungitoxic activity of the seed coat extract seemed to be correlated to the concentration of tannins rather than the two flavonoid standards tested. It was found that at similar concentrations (0.7 g ml^{-1} diluted to 70%), the seed coat extracts of cultivars Encore and PAN311 and kaempferol standard, were fungitoxic to *C. dematium* spores. This indicates that in the pool of soluble phenolic compounds, kaempferol does not play a role in the resistance of the cowpea seed. In other plant species, some polyphenols have been associated with resistance to pathogens such as *Fusarium solani* (Mart.) Sacc. on pea (Kraft 1977, Muehlbauer & Kraft 1978), *P. ultimum* on pea (Muehlbauer & Kraft 1978) and *Colletotrichum graminicola* D. J. Politis on maize (Hammerschmidt & Nicholson 1977). In a study by Sarma *et al.* (2002), chickpea seeds treated with plant growth-promoting rhizobacteria induced production of phenolic compounds. In their study, high concentrations of phenolic compounds were directly linked to resistance of the chickpea to *Sclerotium rolfsii*.

The link between the colour of cowpea seeds and the resistance to the fungal pathogen, *C. dematium*, is reported for the first time. These data could be used to predetermine the resistance of the cultivar to the fungal pathogen before cultivation. Therefore, a further analysis of both soluble and insoluble phenolic compounds on a wider range of cowpea cultivars would be valuable.

5.6 Acknowledgements

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Table 5.1

Characteristics of cowpea seeds used to determine the effect of seed coat colour to resistance or susceptibility to *Colletotrichum dematium*

Cowpea cultivar	Colour	Effect of *Colletotrichum dematium*
CH84	Cream with black eye	Susceptible
Rhino	Brown and cream	Susceptible
PAN 311	Reddish brown	Resistant
Encore	Brown	Resistant

Table 5.2

Total soluble phenolics (mg of galic acid / g of dry weight of extract) obtained from cowpea seeds differing in seed coat colour

		Descriptive statistics					
	Cultivars	Valid N	Mean	Minimum	Maximum	Std. Dev.	LSD (5 level)
Methanol	CH84	9	7.33a	5.4	9.3	1.2	
	Rhino	9	7.38a	3.5	10.0	2.1	
	PAN311	9	8.11a	5.4	10.5	1.8	
	Encore	9	11.03b	8.4	13.6	1.7	1.62
Water	CH84	9	6.95a	3.5	12.5	3.8	
	Encore	9	8.43a	5.0	13.9	3.7	
	Rhino	9	9.10a	7.5	11.0	1.3	
	PAN311	9	15.10b	10.9	19.8	2.7	2.41

Table 3

Anti-fungal activity of different concentration of cowpea seed coat extracts and cowpea flavonoid standards against *Colletotrichum dematium* ("+" for conidium germination and "-" for no germination after 24 h)

Dilution (%)	Water (control)	Kaempferol	Quercetin	CH84	Rhino	Encore	PAN311
100	+	-	-	-	-	-	-
90	+	-	+	+	-	-	-
80	+	-	+	+	+	-	-
70	+	+	+	+	+	-	-
60	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+

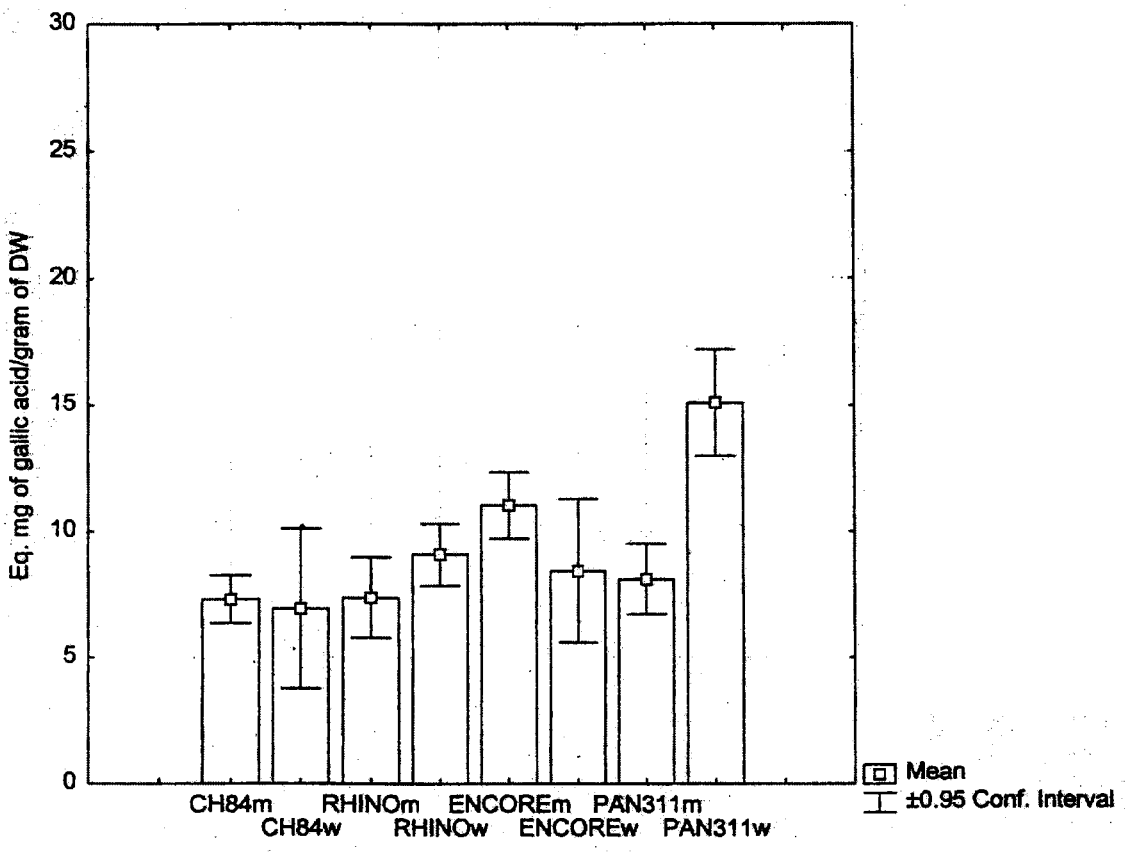


Figure 5.1

Concentration of polyphenolic compounds obtained from seed coat extracts of four cowpea cultivars differing in seed coat colour, extracted with methanol (m) and with water (w)

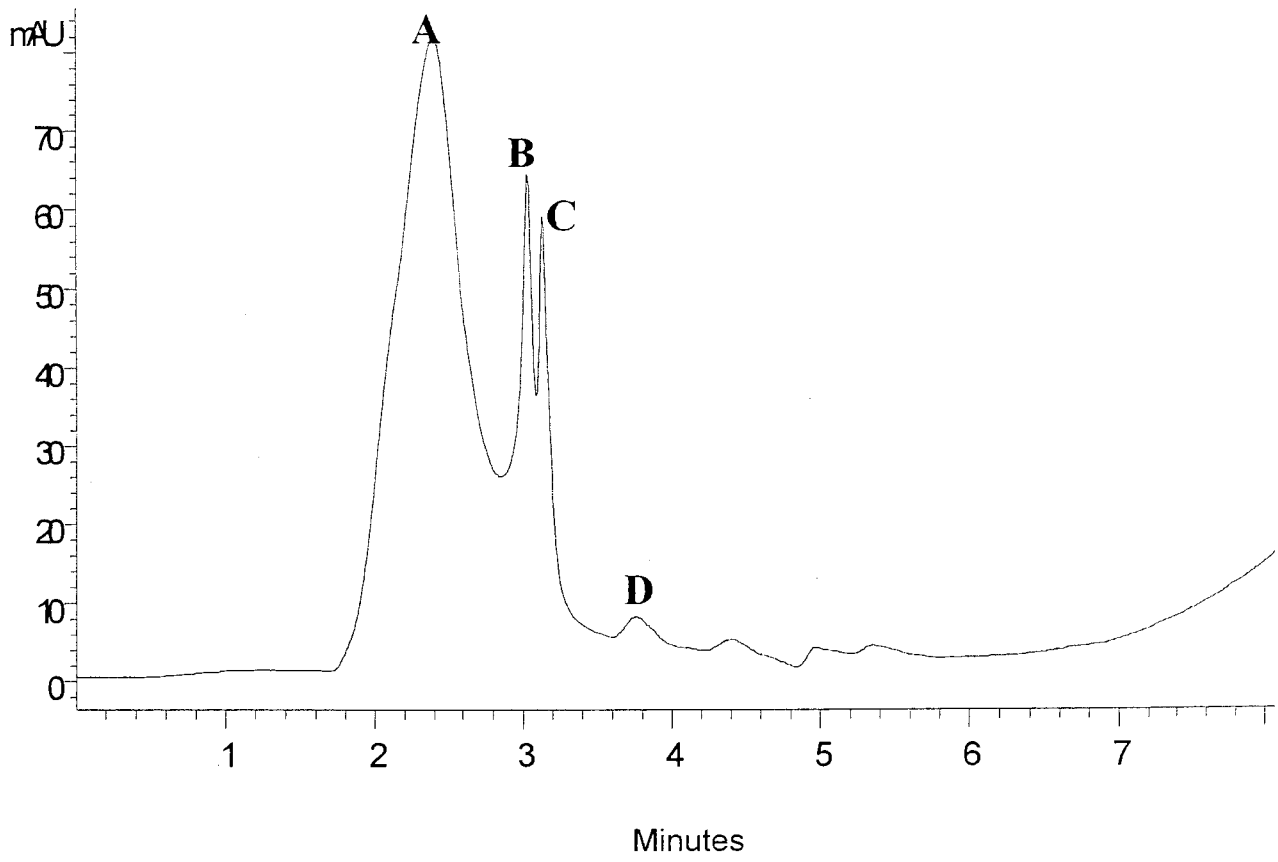


Figure 5.2

An HPLC chromatogram of a crude extract of seed coat of cowpea cv. PAN311, with peakes A, B, C and D, obtained at 280 nm over a 10 min period.

Figure 5.3

Differently coloured seed coats of cowpea stained with vanillin-HCl. A. CH84 shows a uniformly stained seed coat (sc) and an unstained endosperm (E). Rhino seed coat has light and dark red parts (arrows), which correspond, to the brown and cream spots of the seed coat. Both Encore and PAN311 are heavily stained, PAN311 has a thick dark red line (arrow) in the centre of the seed coat. (Bar = 100 μm)

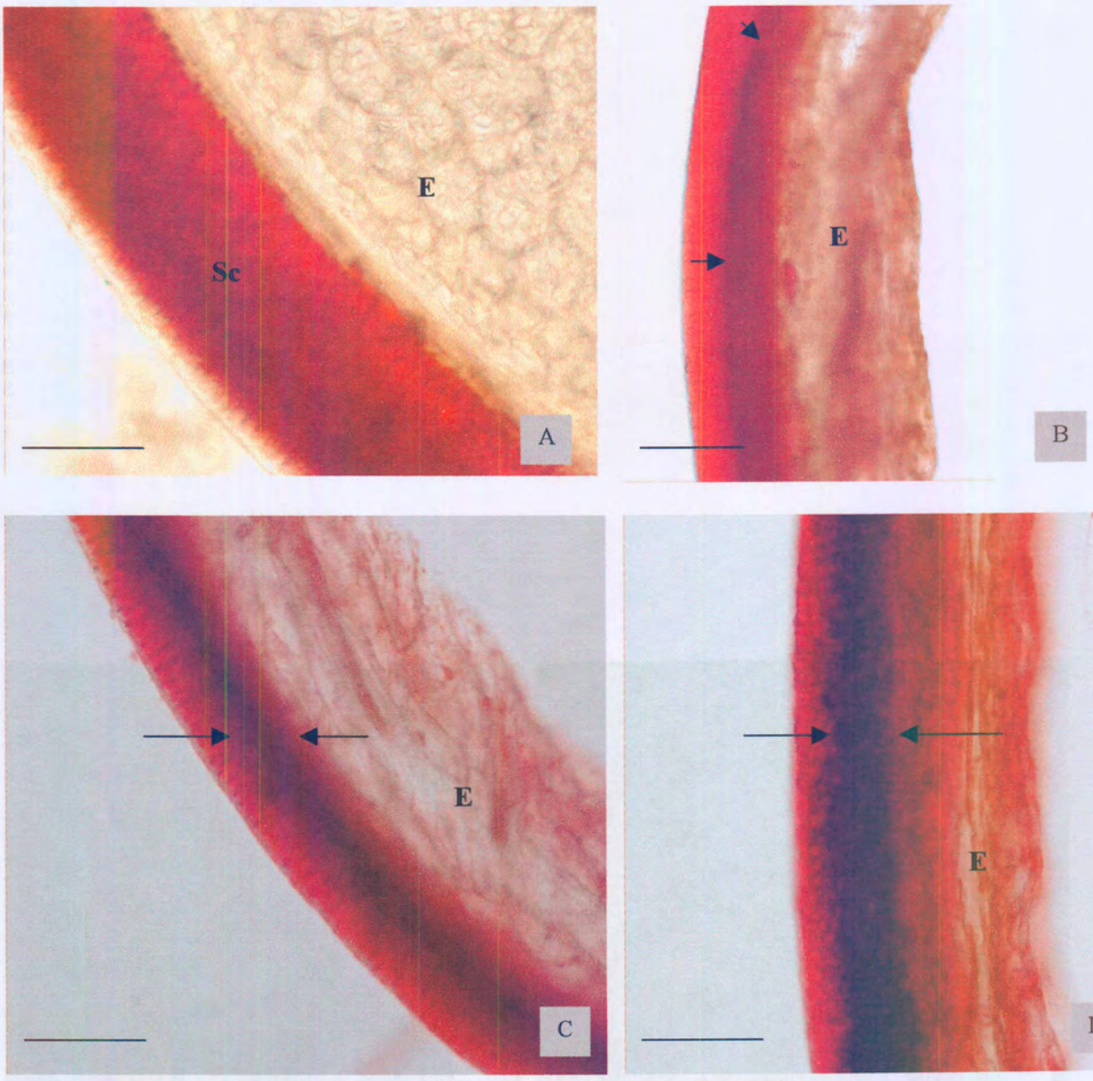
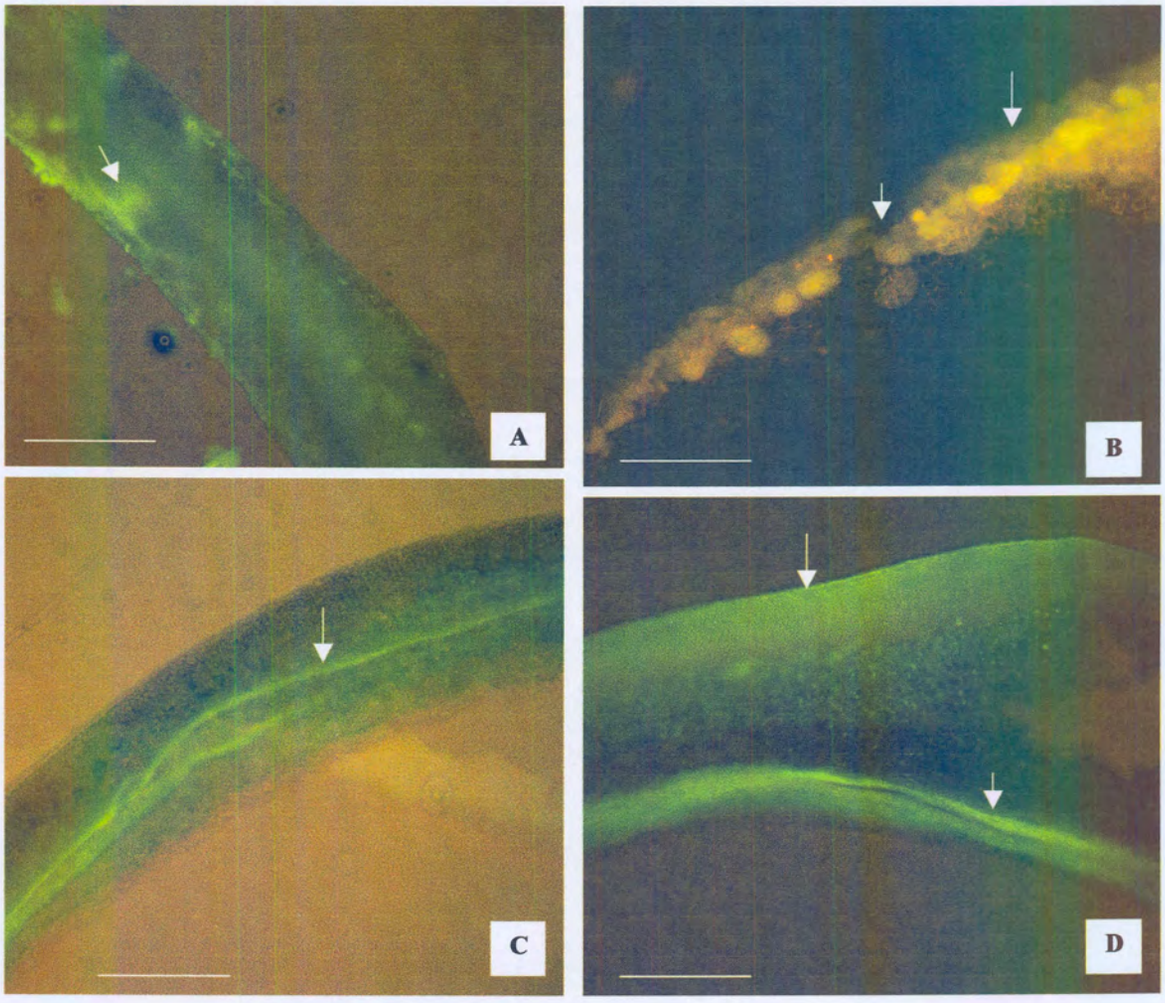


Figure 5.4

Fluorescence microscopy showing cowpea seed coats stained with Neu's reagent and observed under ultra-violet and blue. CH84, susceptible to *C. dematium*, is slightly fluorescent with blue light showing (arrows). Cultivar Rhino has alternating yellow fluorescent spots, indicating the presence of flavonoids. Encore shows fluorescence towards the endosperm. Pan311 shows uniform fluorescence from the edge of the seed coat. (Bar = 100 μm)



CHAPTER 6

Effect of plant age, temperature and dew period on the severity of anthracnose of cowpea caused by *Colletotrichum dematium*

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Pakela Y P, Aveling T A S & Coutinho T A 2002 Effect of plant age, temperature and dew period on the severity of anthracnose of cowpea caused by *Colletotrichum dematium*. *African Plant Protection* 8(1&2) 65–68.

Three factors that influence anthracnose of cowpea caused by *Colletotrichum dematium* were studied in the greenhouse, namely age of the plant at inoculation, incubation period of the pathogen and temperature. Cowpea seedlings were inoculated with a 5×10^5 ml^{-1} conidial suspension of *C. dematium* at three, six and nine weeks after sowing. The inoculated seedlings were maintained in humidity chambers (RH 95 %) for 12, 24 or 36 hours and then transferred to greenhouses at 20, 25 or 30 °C, respectively. Disease severity was rated on a 0–5 scale. Plants inoculated three weeks after sowing were more resistant to infection than those inoculated at six and nine weeks. There were no significant differences in disease severity between plants maintained in humidity chambers for 12 or 24 hours. Cowpea plants were more susceptible to *C. dematium* at the age of nine weeks and maintained at temperatures of 25 and 30 °C for 24–36 hours at high humidity.

Key words: anthracnose, *Colletotrichum dematium*, cowpea, humidity, temperature, *Vigna unguiculata*.

Cowpea (*Vigna unguiculata* (L.) Walp.) is grown in the tropics and subtropics of Africa, India and Asia where leaves, green pods and grain are consumed, especially by resource-poor farmers (Rachie 1985). Anthracnose of cowpea, caused by *Colletotrichum dematium* (Pers.) Grove, was first recorded in India and Malaysia in 1977 (International Mycological Institute 1977), and has recently been reported from the subtropical regions of southern Africa (Smith & Aveling 1997) where mean temperatures during the cowpea growing season range from 6–27 °C. Anthracnose diseases of legumes generally cause severe economic losses in wet, humid, tropical areas (Emechebe & Florini 1997) and are less common in drier savanna regions (Lenne 1992). However, *Colletotrichum truncatum* (Schwein.) and *Colletotrichum capsici* (H. Syd.) F. Butt. & Bisley, causing brown blotch of cowpea, are more prevalent in the latter climates (Emechebe 1981).

Surveys conducted in Botswana, Mozambique and Zimbabwe in 1998 and 1999 during the cowpea-growing season (unpubl. data) indicated that *C. dematium* was more prevalent in monocultured fields than in inter-cropped fields and also in areas of high humidity, especially in Mozambique and Zimbabwe. *C. dematium* was not present in either monocultured or inter-cropped fields in Botswana where temperatures ranged from 28–40 °C during the cowpea growing season.

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Preliminary greenhouse studies furthermore indicated that inoculation with *C. dematium* of cowpea plants younger than three weeks, incubated in a humidity chamber for less than 12 hours after inoculation and maintained at less than 20 °C, resulted in very low disease incidence and severity.

Studies under controlled conditions are essential to provide more specific information on the effects of the environment on disease development. The aim of this study was to investigate the effect of plant age at inoculation, temperature and incubation period (dew period) on infection of cowpea by *C. dematium* in the greenhouse.

Materials and methods

Inoculum preparation

A culture of *C. dematium* (PPRI 6121) originally isolated from cowpea in KwaZulu-Natal (Smith & Aveling 1997), was obtained from the National Collection of Fungi, ARC-Plant Protection Research Institute, Pretoria. Stock cultures maintained at –70 °C in 30 % glycerol were subcultured on potato-dextrose agar (PDA). A conidial suspension was prepared by flooding the surface of a 7-day-old culture with 10 ml sterile distilled water and the concentration was adjusted to 5×10^5 conidia ml^{-1}.

Plant material and experimental design

Cowpea (cv. Rhino) seedlings were reared in 15 cm diameter pots (2 l capacity) in pasteurised

potting soil. Six seeds were planted in each of 216 pots and thinned to four after emergence. Each pot was watered every second day with 200 ml tap water and also received 100 ml Hoagland's solution once a week. The experiment was conducted in three greenhouses with temperatures maintained at 20, 25 and 30 °C, respectively. Each greenhouse was divided into two blocks with 36 pots per block. At 3, 6 and 9 weeks after planting, respectively, the main stems of the plants in nine randomly-selected pots in each block in each greenhouse were inoculated with 2 ml *C. dematium* inoculum per plant using a thistle brush. Plants in the remaining pots served as control and were brushed with sterile water. Three of the nine pots with inoculated plants, and one with uninoculated plants, from each inoculation time within each block at each temperature were placed in a humidity chamber (RH 95 %) for 12, 24 or 36 hours. Thereafter, the plants were returned to the respective greenhouses in which the relative humidity ranged between 55 and 80 %.

Disease assessment

Inoculated plants were assessed for symptom expression seven days after inoculation. The disease scale of Stovold & Smith (1991) was used, where 0 = no symptoms; 1 = few small lesions, 10–20 % stem area infected; 2 = slight, 21–40 % stem area infected; 3 = moderate, 41–60 % stem area infected; 4 = severe, 61–80 % stem area infected with lesions on the leaves; 5 = very severe, >81 % stem area infected with total defoliation and plant collapse being evident or inevitable.

Statistical analysis

A combined analysis of variance (ANOVA) (GenStat 2000) was conducted on disease severity rating over all treatments and means were separated using Fisher's protected least significant difference ($P \leq 0.05$)

Results and discussion

Combined ANOVA indicated that plant age ($P < 0.001$), dew period ($P = 0.041$) and interaction of age and temperature ($P = 0.001$), significantly affected disease severity. The disease was expressed as early as 12 hours after inoculation. There were no significant differences in disease severity between dew periods of 12 and 24 hours and of 24 and 36 hours. However, mean disease rating increased significantly from 1.28 after 12 hours of inoculation to 1.67 after 36 hours.

Studies by Tu (1983) on the effect of precipitation on anthracnose of bean (*Phaseolus vulgaris* L.) caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Scribn., indicated that a wet period of 10 hours was necessary for the fungus to establish infection. In the above experiments, patterns of disease spread were closely associated with heavy rains, with new infections appearing 3–7 days after each rain shower, depending on the temperature. Anthracnose caused by *Colletotrichum coccodes* (Wallr.) S. Hughes on tomato (*Lycopersicon esculentum* Mill.) was more severe when fruit was exposed to continuous wetness (more than 95 % humidity) for more than 10 hours (Dillard 1992). The present greenhouse results showed that a dew period of 12 hours of high humidity was required by *C. dematium* to initiate disease and that extended periods of high humidity promoted infection.

No significant differences in disease severity were evident at three weeks (Fig. 1). At six weeks, disease severities at 20 and 30 °C were similar (1.61) and slightly lower, albeit not significantly (1.28), at 25 °C. At nine weeks, disease severity increased significantly from 20 to 25 °C, and slightly from 25 to 30 °C. There was no significant difference in disease severity between nine-week-old plants maintained at 20 °C (1.25) and six-week-old plants maintained at 25 °C (1.28). Highest disease severity therefore occurred at 25 and 30 °C when plants were nine weeks old.

The cowpea crop is widely grown throughout the tropics and subtropics (Williams 1975) where temperatures range from 15–35 °C. Germination base temperature of cowpea seeds is 8.5 °C, but temperatures above 21 °C are required for vegetative growth and above 30 °C for flowering (Coetzee 1995). Results of the present study indicate that *C. dematium* is able to infect stems of cowpea at 20 °C, but the disease becomes significantly more severe at 25–30 °C. Disease severity tests with other species of *Colletotrichum* indicated that *C. lindemuthianum* (Prasanna 1985), *Colletotrichum truncatum* (Schwein.) Andrus & W D Moore (Emechebe & McDonald 1979; O'Connell et al. 1993), *Colletotrichum acutatum* J H Simmonds and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Zulfiqar et al. 1996), *C. capsici* (Pring et al. 1995) and *Colletotrichum destructivum* O'Gara (Latunde-Dada et al. 1996), are all more severe at temperatures ranging from

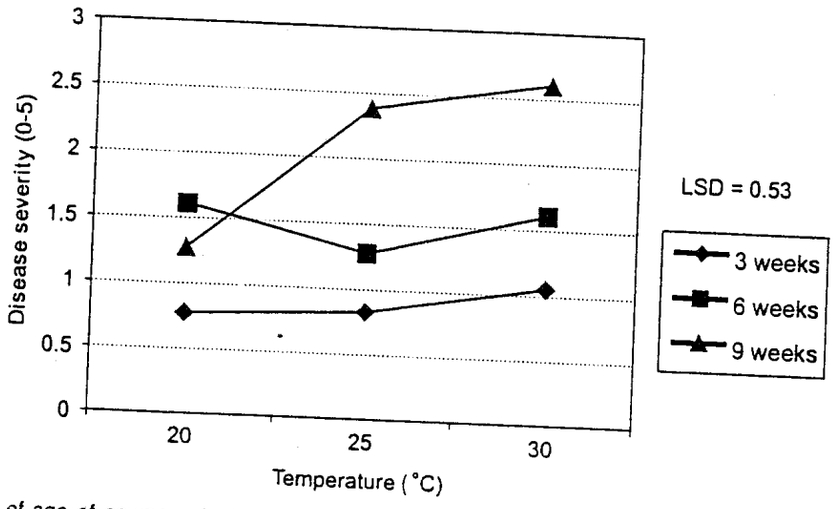

Fig. 1. Effect of age of cowpea plants and temperature on disease severity of *Colletotrichum dematium* in the greenhouse.

22 to 33 °C on their respective hosts.

In conclusion, results obtained in this study confirm those of Gourly (1966), Sutton (1962) and Lenne & Sonoda (1978) implicating *C. dematium* as a pathogen of mature tissue. The study further indicated that *C. dematium* can initiate infection on cowpea at a temperature of 20 °C but was more severe at 30 °C. An incubation period of 12 hours at a high humidity was necessary for infection and disease development and prolonged periods of high humidity resulted in severe infection. These factors are important for predicating disease incidence or severity and should be useful in determining infection by *C. dematium* in the field and in developing disease models and disease forecasting.

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CHAPTER 7

GENERAL DISCUSSION

Although *Colletotrichum dematium* on cowpea was recorded as early as 1977 and 1984 (Lenne 1992), no research was undertaken until 1997 by Smith & Aveling, when the pathogen was discovered for the first time in South Africa. This thesis presents an in-depth investigation on the interaction of cowpea and *C. dematium*. The studies described here were aimed at gaining more knowledge of the biology, pathology, and epidemiology of *C. dematium* on cowpea. As in-depth discussions have been presented in the individual chapters, there is no need to reiterate these deliberations here. Thus, this discussion highlights the most important results of the study.

Isolates of *C. dematium* from infected cowpea fields in southern Africa showed major differences in morphology, pathogenicity and genetic variability (Chapter 3). Morphological differences resulted in 12 groups that differed in colony colours, colony patterns and conidium mass colours. Pathogenicity studies suggested that isolates from Gauteng were the most pathogenic, whereas, those form Zimbabwe were non-pathogenic. Determination of relatedness between isolates with vegetative compatibility groups (VCG) proved to be a major challenge since there are few similar studies undertaken specifically on *Colletotrichum*. The procedure followed was originally formulated for *Fusarium* species (Puhalla 1985). The isolates of *C. dematium* were grouped into three different VCGs based on the size and morphology of the heterokaryon formed. Some isolates straddled VCGs, a phenomenon, commonly termed cross-VCG compatibility. This suggests close relatedness between two VCGs, as was observed in *C. dematium* f.sp. *spinaciae* (Correll *et al.* 1993) and *C. orbiculare* (Wasilwa *et al.* 1993). It is well known in *Colletotrichum* species that relationships among species and their strains are unlikely to be resolved using morphology

alone (Sutton 1992). Morphological plasticity, overlapping phenotypes, low frequency of VCGs (18 or 48 isolates could not be placed in any VCG in this study) made characterisation of these isolates difficult. Isolates were further characterised by random amplified microsatellites polymerase chain reaction profiles (RAMs - PCR). RAMs analysis was able to group together isolates with similar morphological traits, similar pathogenicity levels and isolates of same geographic origin. In one cluster, isolates belonging to VCG1, all from the same area were grouped together. According to Padulosi & Ng (1997) South Africa, particularly Gauteng to KwaZulu-Natal, is one of the areas depicted as a probable centre of speciation of cowpea due to a presence of most primitive wild varieties. Some of these varieties have overlapped geographic areas from South Africa, Swaziland to Zimbabwe and Mozambique. This means that *C. dematium* strains of these areas have evolved together with the particular cowpea variety, and that is the reason why RAMS analysis clusters isolates of the same area together. Also, isolates from the same area tended towards a similar pathogenicity level on the cultivar used in this study. The Zimbabwean strains were non-pathogenic on the cowpea cultivars used here, but did cause disease on the cowpea cultivar from which they were isolated. This was also the same for Mozambican isolates, which were moderately susceptible in the study but were highly pathogenic in the field they were isolated from. This could mean that isolates were host specific and hence couldn't infect the cultivars used in the investigation. This study therefore opens doors to further investigate isolate-variety systems of *C. dematium* on cowpea. The different isolates/strains could be different races of the pathogen.

During infection of cowpea by *C. dematium* (Chapter 4) the extended symptomless biotrophic phase is deceptive to the onlooker because the onset of the necrotrophic phase marks the rapid deterioration of the host tissue. The isolate used in the infection was a virulent isolate. Therefore, the experiment showed the worst-case scenario of the fungus on susceptible stem tissue. It is possible that on leaves, the fungus could be far more damaging. On stem tissue,

the virulent strain of *C. dematium* was found to take less time from initial infection to completely destroying the host tissue as compared to other *Colletotrichum* fungi on leaf material (O'Connell *et al.* 1993, Chongo *et al.* 2002). The combination of subcuticular intramural necrotrophy and destructive necrotrophy make *C. dematium* an effective and aggressive anthracnose fungus.

In the investigation of a relationship between cowpea seed colour and resistance or susceptibility to *C. dematium* (Chapter 5), dark coloured seeds were resistant to *C. dematium*. The amount of total soluble phenolics, the intensity of fluorescence and vanillin-HCl stains proved that dark coloured seeds contained more polyphenols than cream coloured seeds. However, in some cultivars, the colour on the outside of the seed coat does not necessarily guarantee resistance or susceptibility to *C. dematium* as polyphenols might be contained in another layer beneath the visible colour of the seed coat. In some seeds, pigments may be situated in the inner layers of the seed coat while it remains transparent (Harbone 1969). In many instances, dark coloured seeds are known to be resistant to storage fungi (Kritzinger 2000), insects and herbivores (Werker 1997) and imbibition damage (Legesse & Powell 1992) because of their high tannin content. In this study, we can conclude that cream coloured cowpea seeds, which do not have a dark pigment beneath the seed coat are more susceptible to *C. dematium.* The patterns and location of polyphenols on cowpea seeds can thus be used as a tool to predetermine the susceptibility or resistance of the cultivar to *C. dematium*. Dark coloured cultivars should be given priority over white or cream for cultivation by resource poor farmers especially in areas where environmental conditions favour *C. dematium*.

Epidemiology studies (Chapter 6) indicated that *C. dematium* is infectious on cowpea plants aged six weeks and older. Previous studies on *C. dematium* (Sutton 1962, Holliday 1980) reported that *C. dematium* was frequently isolated from mature tissue. Although the fungus can be infectious on three-week-old seedlings (Smith *et al.* 1999), this study showed that the disease severity

increased as the cowpea plant matured. High temperatures and relative humidity are some of the factors that positively influence the severity of the disease caused by *C. dematium* on cowpea. The epidemiological factors that influence the severity of infection of cowpea by *C. dematium* also correlate with environmental conditions occurring during the cowpea season (November-January in South Africa) (Coetzee 1995), i.e. high temperatures (24-32^{0}C), and high rainfall (humidity).

It is possible that an aerial spray can be used shortly before flowering (depending on the cycle of a cultivar) of the cowpea plant, i.e., at four to five weeks when environmental conditions are favourable for the pathogen. This will prevent initial infection of host tissue or reduce the rate of infection on already infected plants before *C. dematium* reaches the necrotrophic phase when profuse sporulation occurs and death of host tissue is inevitable. However, effective prevention methods such as seed treatments, and biocontrol methods should be investigated. The epidemiological factors studied here could be used to formulate a disease forecasting model that will warn the farmer when conditions are favourable for the infection by *C. dematium*. However, as these studies were done in the greenhouse, validation by proper field experiments should be done.

This study revealed some practical answers on the interaction of *C. dematium* and cowpea. Possible further research is required to find a biocontrol agent to control the fungus on cowpea as chemical control is often unsuitable. The risk of using fungicide or pesticide residues could pose a health hazard as the cowpea leaves are consumed as the plant develops. The development of a seed-treatment will also be an advantage to prevent storage fungi as well as expression of seedborne fungi in the field. A disease-forecasting model for *C. dematium* will be useful in determining the period of high disease incidence and infection rate due to high temperatures and humidity. Genetic modification to produce an anthracnose resistant cowpea hybrid is also another possibility.

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SUMMARY

Cowpea (*Vigna unguiculata* (L.) Walp.) is protein-rich basic food legume consumed in many parts of Africa, Asia and Latin America. Susceptibility to a variety of fungal, viral and bacterial diseases and pests is a major limitation to its production and consumption. Anthracnose, caused by *Colletotrichum dematium* (Pers.) Grove, has been identified as a potential threat to small-scale farmers who rely heavily on cowpea production for food. In this study, a total of 48 isolates of *C. dematium* were collected from three cowpea fields in Gauteng and from small-scale farmers in KwaZulu-Natal, Zimbabwe and Mozambique. The identity of these isolates was confirmed using morphological characteristics. Cultural characteristics of these isolates were compared and pathogenicity tests undertaken. The genetic relatedness between collected isolates, based on vegetative compatibility and random amplified microsatellites, was also determined. Cultural characteristics between isolates differed with respect to their colour, zonation and sectoring on potato dextrose agar. There were significant differences in pathogenicity, with isolates from Gauteng and KwaZulu-Natal being highly pathogenic, while most isolates from Mozambique and Zimbabwe were non-pathogenic. Of the 48 isolates collected, 30 were placed into three vegetative compatibility groups. Some isolates were found in more than one VCG. Random amplified macrosatellite polymerase chain reaction profiles clustered isolates with similar morphological characteristics and pathogenicity levels and also from the same geographic area

The infection studies of *C. dematium* on cowpea complemented previous studies by Smith *et al.* (1999). In this study, the emphasis was on the type of necrotrophic infection and formation of acervuli and conidia. To study the necrotrophic phase, stems of 3-week-old cowpea seedlings were inoculated with a conidial suspension of *C. dematium* and incubated for 48, 52, 65, 72, 105 and 120 h. Pieces measuring 1-2 mm were cut from the infection site and fixed in 2.5% glutaraldehyde for 2.5 h. To study the ultrastructure of conidiogenous cells

and conidial formation, samples from culture plates were taken every 6 h and fixed in 2.5 % glutaraldehyde for 1.5 h. Infected material was prepared for viewing with a light microscope, and scanning, high resolution scanning and transmission electron microscopes. The necrotrophic phase observed at 52 hours post inoculation was characterised by rapid destruction of cells from the epidermis extending toward the cortex. Epidermal cells near the secondary hyphae were dissolved while the cuticle of neighbouring cells pulled away from the cell wall. *C. dematium* was found to uses two types of necrotrophic strategies, subcuticular intramural colonisation which was quickly followed by destructive necrotrophy. Lesions were visible at 72 hpi. The stem cuticle remained intact covering the pulvinate acervuli while the remaining tissue had dissolved into a watery mass. During conidial formation, conidiogenous cells differentiated to form elongated conidiophores. Conidia developed from the inner walls of conidiophores and were released together with copious amounts of extracellular matrix.

To characterise the influence of cowpea seed colour on the susceptibility or resistance to *C. dematium*, patterns of polyphenols in the seed coat were characterised. Polyphenols are secondary metabolites that appear to play a vital role in defence of plants against pathogens and predators. They also influence physiological aspects of plants such as seed maturation and dormancy. Localisation and concentration of polyphenols in seeds of four cultivars of cowpea (*Vigna unguiculata*) were determined by a combination of histochemical and biochemical methods. Crude extracts from cowpea cultivars differing in colour [PAN 311 (brown), Encore (reddish-brown), Rhino (mottle brown and cream) and CH84 (cream with brown eye)] were extracted using 50% methanol and boiling water. Determination of total soluble phenolics showed significant differences among the cultivars. In general, the brown cultivars contained more total soluble phenolics than the cream cultivar. On high performance liquid chromatography, kaempferol was detected in all cultivars. The HLPC patterns were similar for all the cultivars. Light and fluorescence microscopy showed

different intensities of red (catechins and tannins) and green or blue fluorescence (flavonoids) directly related to seed coat colour. Seed coats of cultivars PAN311 and Encore emitted higher intensities than Rhino and CH84. To investigate the toxicity of the extracts on the conidia of *C. dematium*, the crude extracts were diluted from 90 to 20%. The undiluted crude extracts (0.7g ml^{-1}) down to 60% dilution of the crude extracts of cultivars PAN311 and Encore prevented germination of *C. dematium* conidia. Therefore, the seed coat extracts of the dark brown cultivars, PAN311 and Encore, showed more resistance to the cowpea pathogen, *Colletotrichum dematium.*

A number of factors are known to influence the success of infection and disease development by fungal pathogens. Three factors that influenced the disease severity of *C. dematium* on cowpea stems were studied. These included the age of the plant at infection, the incubation period of the pathogen on the host and surrounding temperature. Cowpea seedlings were inoculated with a conidial suspension of *C. dematium* at ages 3, 6 and 9 weeks and incubated for 12, 24 or 36 h at temperatures of 20, 25 or 30^{0}C. Disease severity was rated on a 0-5 point scale. Plants inoculated at 3 weeks after sowing were more resistant to infection by *C. dematium* than at 6 and 9 weeks. There was no significant difference between incubating the plants for 12 or 24 h. The interaction of age and temperature indicated that the disease was more severe when the cowpea plants were 9 weeks old and incubated at 30^{0}C. The cowpea plant was found to be more susceptible to *C. dematium* at the age of 9 weeks and at temperatures ranging between 25-30^{0}C for prolonged (24-36 h) high humidity conditions. These results provide essential information on the interaction of *C. dematium,* cowpea and environmental conditions.