A NEW DISEASE OF COWPEA CAUSED BY

Alternaria cassiae

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DECLARATION

I hereby certify that this research, unless specifically indicated to the contrary in the text, is the result of my own investigation and that no part of this thesis has been submitted to any other university.

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SUMMARY

Cowpea (*Vigna unguiculata* (L.) Walp is an indigenous food legume in Africa, which has great production potential, especially in areas with low agricultural resources. During surveys of cowpea fields in southern Africa, a new foliar disease was recorded. *Alternaria cassiae* Juriar & Khan was consistently isolated from diseased plant material. Pathogenicity was confirmed using Koch's Postulates. The effect of different culture media, temperature, light and wounding on the growth and sporulation of the fungus was studied. *A. cassiae* grew well and produced conidia abundantly when maintained on V8-agar at 25°C in a 12h UV-light/12h dark cycle. Sporulation was further enhanced by wounding the cultures. The pre-penetration and infection process of *A. cassiae* on cowpea leaves was studied by light and scanning electron microscopy. Conidia germinated within 2-3h post inoculation (hpi), forming multiple germ-tubes randomly that grew in any direction across the leaf surface. By 8hpi terminal or intercalary appressoria were formed above epidermal cells or over stomata. Occasionally germ-tubes entered stomata, without the formation of appressoria. Penetration of the plant surface, whether directly through the epidermis or indirectly through stomata was observed 72hpi. Following penetration bulbous primary hyphae were observed within the sub-stomatal cavities, secondary hyphae developed from the primary hyphae and grew within the intercellular spaces penetrating epidermis and mesophyll cells. *A. cassiae* is a necrotrophic fungus as the infection process is characterised by a destructive necrotrophic phase where plant cells became necrotic even prior to fungal penetration. Conidial morphology, types and development of the fungus were studied *in vitro* on different culture media and *in vivo* on cowpea leaves. *A. cassiae* produced a mixed population of three conidial types. Conidia were formed singly or in chains of 2-4 conidia. Conidia with long, filiform beaks and conidia with shorter beaks, converted into secondary conidiophores were more frequently produced than mature, beakless conidia on all the media, except on potato dextrose agar. Conidial body and beak sizes were variable when measured in culture and on cowpea leaves. Conidia produced in culture were larger, than those produced *in vivo*. Conidiophores emerged directly through the epidermis or stomata or were formed when hyphae growing on the leaf surface differentiated into conidiophores. Smooth, bud-like conidial initials were produced at the apex of

conidiophores. Conidia matured and became elliptical to obvate and densely verrucose. Once a mature conidium had detached, a small pore was visible at the apex of the conidiophore. *A. cassiae* was shown to be seed-borne in cowpea. Six fungicides i.e. Benomyl, bitertanol, captab, mancozeb, propiconazole and triforine were evaluated for their efficacy in reducing mycelial growth of *A. cassiae in vitro*. All fungicides except benomyl proved to be effective. Cowpea seeds were artificially inoculated with *A. cassiae* and treated with all the fungicides except benomyl. Percentage germination and infection was determined *in vitro*. Percentage emergence, disease incidence, root and shoot lengths and abnormalities were determined in greenhouse trials. Only bitertanol at 1.5x the recommended dosage significantly reduced percentage germination. All treatments except triforine 1.0x and 1.5x significantly decreased the percentage infection of artificially inoculated seeds. None of the treatments except bitertanol 1.5x showed a difference in shoot and root length when compared to the control. Captab 1.5x the recommended rate proved to be the best treatment over all.

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**"Delight yourself in the Lord,
and He will give you the desires
of your heart." Psalm 37:4**

People who make their own rules
when they know they're right…
People who get a special pleasure
out of doing something well (even
if only for themselves)…
People who know there's more to
this whole living thing than meets
the eye: they'll be with Jonathan
all the way.

Jonathan Livingstone Seagull
(Richard Bach)

CONTENTS

ABBREVIATIONS

%	percentage
±	approximately
β	Beta
Σ	Sum of
°C	Degrees Celsius
μm	Micrometers
>	Greater than
a.i.	active ingredient
ARC	Agricultural Research Council
B	Boron
Ca	Calcium
cm	centimetres
cm^3	cubic centimetres
CMA	Cornmeal agar
Co	Cobalt
Cu	Copper
d	day
Fig.	Figure
g	gram
g/l	grams per litre
GCI	Grain Crops Institute
h	hour
ha^{-1}	per hectare
hpi	hours post inoculation
IITA	International Institute for Tropical Agriculture
ISTA	International Seed Testing Association
K	Potassium
kg	kilogram
kg^{-1}	per kilogram
kV	kilovolt
m	metre

Mg	Magnesium
mg	milligram
min	minute
ml	millilitre
ml^{-1}	per millilitre
mm	millimetre
Mo	Molybdenum
no.	number
P	Phosphate
PDA	Potato dextrose agar
ppm	parts per millilitre
PPRI	Plant Protection Research Institute
Rep.	Replicate
S	Sulphur
SDW	Sterile distilled water
SEM	Scanning electron microscopy
t	ton
UV	Ultra-violet
v/v	volume per volume
VOPI	Vegetable and Ornamental Plant Institute
w/v	weight per volume
wk	week
Zn	Zinc

CHAPTER 1

GENERAL INTRODUCTION

Recent studies in South Africa have emphasised the role and potential of indigenous crops in providing many of the basic needs of resource poor communities (Anon. 1996), and also to serve as rich sources of genetic material from which selections can be made for possible commercial utilisation. Producing quality food in increasing quantities provides an enormous challenge to agriculturists. It is of vital importance to increase the output of cheaper sources of quality protein, particularly in small-scale farmer communities (Anon. 1996). There is also a great need to develop crops from appropriate indigenous plants, which are eminently well suited for cultivation in the large areas of Southern Africa, where often a low agricultural potential prevails due to a low unreliable rainfall, harsh conditions and the low income of resource poor farmers (Quass 1995). Many developed and developing countries have started to introduce the use of indigenous food crops as dietary supplements and animal fodder (Coetzee 1995). Even though various studies in South Africa have emphasised the importance of indigenous plants, there is however, still difficulty in the cultivation and management of these indigenous crops.

Small-scale and emerging farmers have started growing various indigenous crops such as bambara groundnut (*Vigna subterranea* (L.) Wilcz), pigeon pea (*Cajanus cajan* (L.) Huth), amaranth (marog) (*Amaranthus* spp. L.) and cowpea (*Vigna unguiculata* (L.) Walp) (Anon. 1996). However, when crops are grown in monoculture and in closer proximity to one another, diseases become a potential threat (Agrios 1988). Most of the research done until now in South Africa has concentrated on the commercially important exotic crops. Little research has been done on indigenous crops and the diseases affecting them.

Cowpea is an important food legume crop in Africa, south of the Sahara and particularly the west African savannah zones (Kay 1979; Coetzee 1995) and was identified as an important indigenous food and fodder crop in South Africa with numerous uses and the potential for low-cost production. Since little is known of the diseases that threaten this crop, a collaborative program between the University of Pretoria and the Agricultural Research Council (ARC) - Vegetable and Ornamental Plant Institute focussing on cowpea diseases, was established.

A survey of cowpea fields in Southern Africa revealed the presence of a destructive new foliar disease (Chapter 3). Isolations were made from diseased material collected during the survey, Koch's postulates were proved and the causal pathogen was identified as *Alternaria cassiae* Juriar & Khan. The aim of this chapter was to describe this new foliar disease and to develop a disease rating scale that could be used to determine percentage disease severity in a cowpea field. A Disease Note entitled "First report of *Alternaria cassiae* on cowpea" has been published in Plant Disease.

In Chapter 4, the manuscript titled "Effect of culture media and other factors on the growth and sporulation of *Alternaria cassiae*" has been submitted as a Short Communication to the South African Journal of Botany.

As yet, there has been only one study on the infection process of *A. cassiae* on sicklepod (*Cassia* spp.) (Van Dyke & Trigiano 1987), but none on the infection process of *A. cassiae* or any other *Alternaria* spp. on cowpea. The interaction between cowpea and *A. cassiae* was therefore studied using light microscopy and

scanning electron microscopy (SEM). The aim of this study was to determine the various steps in the infection process and structures formed during and after penetration (Chapter 5). Chapter 5, entitled "Infection studies of *Alternaria cassiae* on cowpea" has been submitted to the Canadian Journal of Botany to be considered for publication.

In Chapter 6, the manuscript titled "Conidial morphology and development of *Alternaria cassiae*" has been submitted to the European Journal of Plant Pathology. This chapter reports on the conidial types and morphology of *A. cassiae in vitro* and *in vivo* as well as the conidial development of the fungus on cowpea leaves, using light microscopy and SEM.

Neergaard (1977) stated that most *Alternaria* spp. are seed-borne. The aim of this study was also to determine whether *A. cassiae* is seed-borne on cowpea and if so, various seed treatments would be evaluated for their efficacy in reducing *A. cassiae* on cowpea seed. The ability of the treatments to inhibit growth of the fungus *in vitro* and *in vivo* would be tested, as well as the effect of the treatments on germination (Chapter 7). Chapter 7, entitled "The evaluation of six fungicides for reducing *Alternaria cassiae* on cowpea seed" has been submitted to Plant Disease to be considered for publication.

This research aims at providing information on *A. cassiae*, the causal agent of a new foliar disease of cowpea.

The reader will notice some inconsistencies in author citation, manuscript compilation and referencing. Since manuscripts were submitted to different journals and had to comply with different requirements, such inconsistencies were unavoidable. Chapters 1, 2, 3 and 8 have been prepared according to the guidelines of the South African Journal of Botany. The reference lists for Chapters 1 and 8 have been combined at the end of Chapter 8.

CHAPTER 2

LITERATURE REVIEW

2.1   THE HOST: *Vigna unguiculata* (L.) Walp

2.1.1   Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is a traditional and indigenous crop in Africa and possibly, one of the oldest crops utilised by man (Coetzee 1995). During the past five years, cowpeas have been the subject of many research projects carried out by the Agricultural Research Council, Roodeplaat - Institute for Vegetable and Ornamental Plants. Cowpeas are increasingly important as a human dietary supplement and stock feed and may also provide a possible source of income (Coetzee 1995).

2.1.2   Origin and distribution

Cowpeas are of ancient cultivation and have probably been used as a crop plant since Neolithic times (Chevalier 1944). Due to lack of archaeological evidence the centre of origin of cowpeas is uncertain and has been previously reported as possibly Asia (Faris 1965), Africa (Piper 1913; Anderson 1952; Burkhill 1953), Persia (Wight 1907) or even South America (Piper 1913). According to Summerfield *et al.* (1974), Sauer (1952) and Steele (1972) proposed a solely Ethiopian centre of origin and suggested that cultivated cowpeas were domesticated there with sorghum. Burkhill (1953) concluded that cowpea is an ancient crop and came to Europe early enough for the Greeks and Romans to grow it under the name *Phaseolus*. Vavilov (1950) partly agreed with Wight (1907), proposing an Asiatic origin and considering China and Abyssinia as secondary centres of origin.

Cowpeas could have migrated along the coastal and Indian trade routes, though at present it is impossible to be certain whether the migration started in Africa or Asia (Sauer 1952; Mehra 1963; Singh *et al.* 1971).

Purseglove (1968) reported that the wild *V. unguiculata* is widespread in tropical Africa and that it seems reasonable to assume that the crop was domesticated in this region and that it spread from tropical Africa in remote times through Egypt or Arabia to Asia and the Mediterranean. Cowpeas reached Europe from Asia and perhaps from North Africa before 300 BC, and the Spanish took the crop to the West Indies in the seventeenth century AD (Purseglove 1968; Steele & Mehra 1980). The crop probably reached the United States in the early eighteenth century (Wight 1907; Purseglove 1968). The distribution of cowpea to other areas of the world took place through migration and along trade routes. Cowpea is now common in the tropics, subtropics and an important food legume crop in Africa, south of the Sahara particularly in the west African savannah zone (Kay 1979; Coetzee 1995). According to Ehlers & Hall (1997), about 66% of the production and more than 75% of the area of production is spread over vast Sudan Savannah and Sahelian zones of sub-Saharan Africa from Senegal going east through Nigeria and Niger to the Sudan, in Kenya and Tanzania, and from Angola across Botswana to Mozambique. Substantial quantities of cowpeas are also produced in South America, Asia and the south-eastern and south-western areas of North America. Ehlers & Hall (1997) further reported that cowpeas are grown on approximately 7 million ha in warm to hot regions of the world. In 1981, 16 African countries produced 66% of the world yield (Coetzee 1995). Nigeria is reported to be responsible for approximately 70% of the world cowpea production (Blade *et al.* 1992).

Cowpeas are widespread throughout southern Africa (Coetzee 1995). In South Africa cowpeas are mainly produced by small-scale farmers as a human food source and on a larger scale as an animal fodder. Production areas are confined to the Northern Province, North-West Province, Mpumalanga, Gauteng and KwaZulu Natal (Coetzee 1995; Van den Heever *et al.* 1996).

2.1.3 Nomenclature

Vigna unguiculata (L.) Walp.

<u>Family:</u>   Fabaceae

<u>Synonyms:</u>   *Dolichos unguiculatus* L.

              Dolichos catjung Rurm.

              Phaseolus cylindricus L.

              Vigna cylindrica (L.) Skeels.

              Vigna catjung Burm

              Vigna sinensis var. *cylindricus*

(Tindall 1983)

<u>Common names:</u>   English – cowpea, bachapin bean, black eye bean or pea, catjang china pea, cowgram or southern pea

                   Afrikaans – akkerboon, boontjie, koertjie or dopboontjie

                   Lovedu – dinawa ja badogwa, dinawa ja thsekene

                   Ndebele – dinawa

                   Shona – muriwo we nyemnba

                   Thonga – mbawen nyangana

                   Shangaan – dinaba, munaoa, tinyawa

Tswana – dinawa, nawa –ea-setswana

Venda – munawa (plant), nawa (fruit)

Zulu – imbumba, indumba, isihlumayo

(Kay 1979; Fox & Norwood Young 1982; Tindall 1983; Van den Heever *et al.* 1996)

2.1.4   Botanical description

Purseglove (1968), Johnson (1970), Kay (1979) and Fox & Norwood Young (1982) described cowpea as follows:

An erect, trailing, climbing or bushy herbaceous summer annual.

Roots: Stout tap-root, with numerous lateral spreading branches. Penetrates up to four feet (1.2m) in the soil.

Stems: Very variable, usually procumbent. Striate, smooth or slightly hairy and sometimes tinged with purple, 0.9–1.2m long in the upright types and 1.2–1.8m long in the spreading types.

Inflorescence: Flowers are arranged in racemose or intermediate inflorescence at the distal ends of 5-60cm long peduncles. Flowers are borne in alternate pairs usually with two to a few florets. They are conspicuous and may be white (Fig. 2.1), dirty yellow, pink, pale blue or violet. Flowers are self-pollinating and borne on short pedicles. Flowers open early in the day, close at approximately mid-day and then wilt to collapse.

Leaves: Alternate and trifoliate. Leaflets are usually dark green, large (6-16 x 4-11cm) and ovate to lanceolate. Leaf petioles are 5-25cm long and grooved with inconspicuous stipels.

<u>Fruit:</u> Pods vary in size, shape, texture and colour. They may be erect, coiled or crescent-shaped, 3-23cm long. Pods are green and usually become yellow when they ripen, but may also be brown or purple.

<u>Seeds:</u> Usually 8-20 seeds per pod. Seeds vary considerably in size, shape and colour (Fig. 2.2). They are relatively large (2-12mm long) and weigh 10-25g per 100 seeds. The testa may be smooth or wrinkled and white, buff, green, brown, red and purple to black or often mottled.

Fig. 2.1   A white cowpea flower.

Fig. 2.2   Various colours of cowpea seed.

2.1.5 Cultivation conditions

Cowpeas can be cultivated under a wide range of conditions. Although they are sensitive to cold and are killed by frost (Purseglove 1968), cowpeas are generally regarded as a heat-loving, drought-resistant crop, which can be grown with less rainfall and under more adverse conditions than *Phaseolus vulgaris* L. (green bean) (Purseglove 1968; Johnson 1970; Kay 1979). A minimum temperature of 9°C is required for seed germination; therefore the earliest possible planting date is when night temperatures exceed 9°C (Coetzee 1995; Quass 1995). Temperatures between 20 and 35°C are conducive to vegetative growth, while temperatures above 33°C advance flowering time, but can cause flower abscission and poor fruit set if in combination with moisture stress (Kay 1979; Coetzee 1995).

Cowpea is grown successfully on a wide range of soils, provided the soil is well-drained as the crop is sensitive to water-logging and should not be planted in lands which easily become water-logged, as this will affect the growth of roots and the development of *Bradyrhizobium* bacteria (Kay 1979; Coetzee 1995; Quass 1995). In general, the heavier loams are probably best for cowpea grain production and, where light sands are used, the use of compost or manure to improve the retention can be recommended. Cowpeas are tolerant of slightly acid soil pHs (pH 5.5-6.0), but alkaline soil causes a drop in nodulation and an increase in leaf chlorosis (Johnson 1970; Coetzee 1995; Quass, 1995). The ideal conditions would appear to be ample soil moisture with low atmospheric humidity on a well-drained soil (Johnson 1970). With regard to fertilization, good results have been obtained with rotational cropping with maize [*Zea mays* (L.)] and other crops as cowpea are able to utilise the remaining

fertiliser (Quass 1995). According to Coetzee (1995), good drainage, a neutral pH, adequate Ca, Mg, K, P, and S as well as available B, Co, Cu, Mo, and Zn result in good development of *Rhizobium* nodules and thus efficient nitrogen fixation, and optimal plant growth. Fertilization should only be considered to prevent poor seedling growth, which may occur shortly after emergence due to a shortage of nitrogen (Quass 1995). For sandy soils 0-15kg nitrogen ha^{-1} is recommended and for clay soils 0-5kg nitrogen ha^{-1}. Phosphorus recommendations for soils with a low phosphorus status are 10kg ha^{-1}, while 5kg ha^{-1} is recommended for soils with a high phosphorus status (Quass 1995). Excessive application of P, or planting on soils with a high phosphorus status, could lead to excessive uptake of this element. This could promote flower abscission during stress conditions and impede seed set (Coetzee 1995). Fertiliser should not be placed with the seed. Potassium fertilization is not necessary except if the soil contains less than 80mg of potassium kg^{-1} soil (Quass 1995).

Water requirements of cowpeas vary according to their growth habit. The water requirement of cowpea types with a determinate growth habit, decline rapidly and considerably after the flowering stage, and a total consumption of 140mm soil water in a 66 day growing period was measured (Coetzee 1995). Cowpea types with an indeterminate growth will use more soil water over a longer period of time (Coetzee 1995). In South Africa an even distribution of as little as 300mm rain per annum can produce a satisfactory crop of 6t ha^{-1} hay and 1t ha^{-1} seed (Quass 1995).

2.1.6 Planting and harvesting procedures

The optimal planting date varies according to the cultivar. In general, the best planting times in South Africa are mid-November in the cooler areas and mid-December in the warmer areas. Cowpeas can also be planted at the beginning of the rainy season (early October) or late in January, but at a much higher risk. Planting too early could cause poor germination and flower abscission in the flowering period (December/January). Planting too late, on the other hand, can cause losses as a result of early frost, or there may not be enough time for the completion of pod formation. This could lead to a loss in grain yield and material, and can affect crude-protein content (Quass 1995). Cowpea seeds are sown 2-3 per hole, with a plant depth of 2-5cm in moist soil. According to Quass (1995), under South African conditions the erect types perform much better in narrower rows (0.9m rows; 111 000 plants ha^{-1}). The semi-spreading and spreading types do better in wider rows (1.5m rows; 66 000 plants ha^{-1}). A spacing of 10cm between plants in the row is ideal (Quass 1995).

The average number of days from planting to various growth stages (Table 2.1) is influenced by factors such as cultivar, planting date, temperatures during the growing season, soil type and rainfall.

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Table 2.1. Days from planting to the respective growth stages of cowpea types.

Growth stage	Growth habit	Number of days (±)
50% flower	Runner	60
	Upright	50
Ready for making hay	Runner	120
	Upright	100
Ready for harvesting of grain	Runner	140
	Upright	120

(Quass 1995)

According to Quass (1995), the time of harvesting as well as the harvesting procedures depend on the cultivar and the purpose for which the plants were planted. The best time to harvest, in the case of grain production, is when 95% of the pods have dried off. Upright or semi-upright cultivar types can be pulled up and threshed by making slight adjustments to machinery used for dry beans. A commercial harvester can be used when pods are borne higher on the stems. Good quality hay is harvested when the first 10% of pods show discolouration but have not yet dried off. In both cases harvesting is best postponed in wet weather (Quass 1995).

Cowpea is a candidate for intercropping and crop rotation, due to the N-fixing ability. According to Blade *et al.* (1992), Arnon (1972) reported that 98% of cowpea grown in Africa is intercropped.

Cowpeas can be intercropped with taller plants such as maize, particularly in high rainfall areas due to their exceptional shade tolerance (Johnson 1970). In high rainfall areas like Nigeria, cowpea is generally intercropped with cassava (*Manihot esculenta* Crantz.) and/or maize and in semi-arid savannah regions, cowpea is grown with sorghum (*Sorghum bicolor* L. Moench) or pearl millet (*Pennisetum glaucum* L. R. Br.). Some cowpea lines have potential under different levels of management, while others show a drop in yield when they are intercropped (Blade *et al.* 1992).

Average grain yields of 224-760kg ha^{-1} for African countries have been reported in literature (Johnson 1970; Summerfield *et al.* 1974). However, far higher yields have been reported from large-scale, intensively managed systems. In South Africa, grain yields of 896-1120kg ha^{-1} have been obtained (Van den Heever *et al.* 1996). If insect pests are controlled successfully, yields may be even higher. Grain yields of 880-3024kg ha^{-1} have been recorded in California (Summerfield *et al.* 1974). For the production of hay, approximately 6t ha^{-1} can be expected under South African conditions (Van den Heever *et al.* 1996). Fresh leaf yields for vegetable use can range from 0.1-0.4t ha^{-1}. Leaves for fresh use are only picked up to the 50% flowering stage (Van den Heever *et al.* 1996).

2.1.7 Uses

The main use of cowpea is as a food legume, especially for small scale farmers and communities in rural areas (Kay 1979; Coetzee 1995). Cowpea is a very palatable,

highly nutritious crop and is relatively free of metabolites or other toxins (Kay 1979; Quass 1995).

The chemical composition of cowpea corresponds with that of most edible legumes (Table 2.2), and the use of cowpea as a vegetable provides an inexpensive source of protein in the diet. The seeds also contain small amounts of β-carotene equivalents, thiamin, riboflavin, vitamin A, niacin, folic acid and ascorbic acid (Kay 1979; Tindall 1983).

Table 2.2. Chemical composition of cowpea (%).

	Leaves	Seeds	Hay
Protein	4.7	22-24	18
Carbohydrate	8	56-66	-
Water	85	11	9.6
Crude fibre	2	5.9-7.3	23.3
Ash	-	3.4-3.9	11.3
Fat	0.3	1.3-1.5	2.6
Phosphorus	0.063	0.416	-
Calcium	0.256	0.104-0.76	-
Iron	0.005	0.005	-

(Kay 1979; Tindall 1983; Quass 1995)

In Africa, the young leaves are eaten as spinach or dried for use in soups, while the haulms are fed to livestock (Allen 1983). According to Kay (1979) and Quass (1995), the seeds are often cooked together with other vegetables to make a thick soup, or grounded and the meal used to make cakes. Fresh mature pods may be boiled as a vegetable. Dried leaves are preserved and sometimes eaten as a meat substitute (Fox & Norwood Young 1982; Quass 1995). According to Kay (1979), in some countries e.g. India, cowpeas are grown as dual-purpose crop. Green pods are used as vegetables and the remaining parts as livestock fodder. In advanced agriculture, cowpeas are used as a cover or anti-erosion crop (Allen 1983). The cowpea crop, when managed well, has the potential to fulfil most of the basic needs of rural communities.

2.1.8 Pests and diseases

Low yields of cowpea can be attributed to diseases, pest damage and soil fertility problems. Cowpeas are susceptible to a very wide range of pests and pathogens, which attack the crop at all growth stages (Allen 1983). These include insects, bacteria, viruses and fungi. However, this chapter will only report on those fungal pathogens of importance to this thesis. Some 40 species of fungi are cowpea pathogens, and as with other crops, the economic importance of cowpea pathogens also varies considerably with ecological zone and environmental conditions (Allen 1983).

Emechebe & Florini (1997) reported that the following fungal diseases were of importance in Africa.

- Septoria leaf spot (*Septoria vignae* P. Henn and *S. vignicola* Vasant Rao, S. Kosopolzanii Nikolajeva

- Scab (*Sphaceloma* sp.)

- Brown blotch (*Colletotrichum capsici* (Syd) Butler and Bisby and *C. trancatum* (Schwein.) Andrus and W.D. Moore)

- Cercospora leaf spot (*Cercospora* spp.)

- Ashy stem blight (*Macrophomina phaseolina* (Tassi) Goid.)

The following pathogens causing foliar diseases on cowpeas have been reported in South Africa:

- *Ascochyta pisi* Lib.   (Doidge 1924)

- *Alternaria cassiae* Juriar & Khan   (La Grange & Aveling 1998)

- *Cercospora cruenta* Sacc.   (Doidge 1924)

- *Colletotrichum lindemuthianum* Sacc. & Magn. (Doidge & Bottemley 1931)

- *Colletotrichum dematium* (Pers.) Grove   (Smith & Aveling 1997)

- *Septoria vignae* P. Henn.   (Gorter 1977)

- *Rhizoctonia solani* Kühn   (Doidge 1924)

- *Uromyces appendiculatus* Pers.   (Doidge *et al.* 1953)

At least eight fungal pathogens have been recorded on cowpea in South Africa, some of them causing more severe symptoms than others and also differing in virulence due to different environmental conditions. In South Africa, cowpeas are playing an increasingly important role in providing dietary needs to small-scale farmers. However, when cowpeas are grown in greater abundance and in monoculture these diseases may pose an even more serious threat. This reinforces the need for research on the diseases of cowpea.

2.2   THE PATHOGEN: *Alternaria cassiae* Juriar & Khan

2.2.1   Introduction

Alternaria cassiae Juriar & Khan is a dematious hyphomycetous fungus originally isolated from *Cassia holsericea* Fresen. (sicklepod) in Pakistan (Juriar & Khan 1960). This plant pathogenic species is commonly used as a biological control agent for sicklepod (Walker & Riley 1982; Walker & Boyette 1985). Until recently, *A. cassiae* has mainly been associated with species of *Cassia* (David 1991). During field surveys in Botswana and the Mpumalanga and Gauteng provinces in South Africa in the 1998 growing season, *A. cassiae* was identified as being the causal pathogen of a new fungal leaf disease on cowpea (*Vigna unguiculata* (L.) Walp) (La Grange & Aveling 1998).

2.2.2   History and nomenclature

Alternaria cassiae Juriar & Khan

Alternaria sp. is a dictyosporic genus of the family Dematiaceae, order Hyphomycetes, Fungi Imperfecti.

The genus *Alternaria* was founded by Nees in 1817, with *A. alternata* (originally *A. tenuis*) being the type and only species described (Elliot 1917; Wiltshire 1933). The dispute over the taxonomic position of *Alternaria* started in the early 1800's, when Fries (1832) described the genus *Macrosporium* and differentiated it from

Cladosporium, *Helminthosporium*, and *Sporodesmium*. All the "*Alternaria*-like" specimens were grouped together in the genus *Torula* (Rotem 1994). After several researchers questioned this assignment, Fries acknowledged the existence of the genus *Alternaria*, but found that it differed from *Macrosporium* (Tweedy & Powell 1963). Elliot (1917) considered *Alternaria* and *Macrosporium* to be distinct genera. However, Angell (1929) found them similar and used the epithet *Macrosporium* to designate both groups. Wiltshire (1933) resolved the confusion when he agreed that *Alternaria* and *Macrosporium* are congeneric, but he considered *Alternaria* to be a more appropriate epithet for designating the genus. The epithet *Macrosporium* became the *nomen ambiguum*. *Alternaria* spp. may be segregated into several groups according to catenulation (the number of spores in the chain) (Rotem 1994). Neergaard (1945) distinguished between 1.) *Longicatenatae* (conidia in long chains of 10 or more; conidia either beakless or with very short beaks); 2.) *Brevicatenatae* (short chains of 3-5, conidia have fairly short to fairly long beaks) and; 3.) *Noncatenatae* (conidia are usually formed singly and may be beakless, but usually have long beaks). According to Rotem (1994), *A. cassiae* is grouped under the *Noncatenatae*.

According to Simmons (1982), several collections of *Alternaria* of the "*A. alternata* group" (previously known as *Macrosporium*) have been described and reported, often as novel species, from pods and branches of *Cassia* species in the Americas, e.g. *Macrosporium cassiaecolum* Thümen (1879), *M. leguminum* Cooke (1878), *M. guaraniticum* Spegazzini (1886) and *M. ravenelii* Thümen (1882). However, none of these names are currently associated with the literature on pathology of leguminous plants (Simmons 1982).

Juriar & Khan originally isolated *A. cassiae* in 1960, from *C. holsericea* in Pakistan. Late in 1980, an *Alternaria* isolate from a diseased seedling of *Cassia obtusifolia* L. was identified. Even though the conidium measurements of this new isolate did not identically match the type description of *A. cassiae*, the conidia revealed the same distinctive pattern of development. It was therefore equated under the name *A. cassiae* (Simmons 1982). Conclusive support for the above decision was obtained when a diseased leaflet from *Cassia* sp., bore conidia almost identical to the type description of *A. cassiae* (Simmons 1982).

2.2.3 Morphology and cultural characteristics

The mycelia of *A. cassiae* are composed of branched, septate, pale brown to almost hyaline hyphae (David 1991). Isolates grow readily on PCA, V8-agar and Hay medium (Simmons 1982). Colonies on PCA are dark fuscous-brown, zonate with grey-white aerial mycelia (David 1991). According to Simmons (1982), sporulation is particularly abundant on V8-agar held in a light-dark cycle, or after scarification of surface mycelia. When colonies are left undisturbed, the dark brown-black mycelia tend to develop an overlay of pale grey non-sporulating hyphae (Simmons 1982). Conidiophores are straight, curved or geniculate and arranged in bundles (David 1991). Several conidiogenous loci are present and conidiophores may be septate, branched or unbranched and 36-112 x 4-5.5 μm in size; the cell walls are generally smooth but occasionally slightly verrucose (David 1991). In culture the conidiophores are produced as lateral extensions of the mycelia. Conidia of *A. cassiae* are large and darkly pigmented (Mims *et al.* 1997). Light to dark-brown

conidia are obclavate, muriform and borne singly or in short chains of 2-4 conidia (Simmons 1982; David 1991). Each conidium is divided by (7-12) transverse and (3-7) longitudinal septa into multiple compartments (Simmons 1982; David 1991; Mims *et al.* 1997). The young, narrowly obvate conidium may become 55µm and longer, before transverse septation is initiated (Simmons 1982). Three to six transverse septa are produced concurrently with beak initiation. Lengthening of the beak and addition of transverse and a few longitudinal septa follow (Simmons 1982). The *A. cassiae* conidium body retains a long elliptical to narrowly obvate shape, with sizes ranging from 65-90 x 20µm (Simmons 1982; David 1991). According to Mims *et al.* (1997), most mature conidia measure 20-25µm in width, and excluding the length of the beak, about 80-85µm in length. The conidial beak of *A. cassiae* is aseptate, initially filiform, reaching up to 200µm in length when produced in culture, but a length of 100-125µm is more usual (Simmons 1982; David 1991).

According to Simmons (1982), the population of beaked conidia, even in very young colonies, becomes mixed. Solitary erostate conidia, as well as erostate conidia that have generated secondary conidiophores from lateral conidial cells are present in the same population. The conidia generating secondary conidiophores consequently give rise to chains of 2-4 conidia. The tip of a filiform beak may sometimes be converted into a slightly enlarged functional secondary conidiophore (Simmons 1982), or the beak may be absent altogether (David 1991). The sporulation potential of this species is so great that conidia occasionally produce an apical filiform beak and a secondary conidiophore at essentially the same time (Simmons 1982). Conidia are produced tretically from the conidiogenous loci, which are arranged sympodially on the conidiophore (David 1991). Cultures of *A. cassiae* maintained on V8-agar contain a

conidium population with a mixture of conidia with filiform beaks, of others at full size, septation and colour but without beaks, and of conidia with apical (and sometimes basal or lateral) conidiophores and secondary conidia in chains (Simmons 1982).

2.2.4 Hosts

The host range of *A. cassiae* is limited. It is most commonly found on *Cassia* species, including the following: *C. angustifolia* Vahl, *C. fistula* L., *C. holosericea*, *C. obtusifolia*, *C. occidentalis* L. and *C. sophera* L. Wall. (David 1991). The fungus has also been isolated from *Albizia procera* (Roxb.) Benth, *Bauhinia purpurea* L., *Crotolaria spectabilis* Roth and *Rhynchosia* sp. (David 1991), and from cowpea (*Vigna unguiculata* (L.) Walp) (La Grange & Aveling 1998).

2.2.5 Geographical distribution

A. cassiae has been found in the following regions:

Africa:	South Africa	(David 1991; La Grange & Aveling 1998)
North America:	USA (Florida)	(David 1991)
Southern United States:		(Van Dyke & Trigiano 1987)
Asia:	India and Pakistan	(David 1991)

2.2.6 Disease symptoms

The Alternaria diseases are among some of the most common diseases of plants throughout the world. They primarily affect the leaves, stems, flowers and fruits of annual plants, especially vegetables and ornamentals (Agrios 1988). Alternaria diseases usually appear as leaf spots and blights, but may also cause collar rots, damping-off of seedlings and tuber and fruit rots. In general, leaf spots caused by *Alternaria* spp. are dark brown to black, and enlarging, usually developing concentric rings, which give the spots a target-like appearance (Agrios 1988). The pathogen usually attacks the lower, senescent leaves first, the disease progresses upwards, leaves become chlorotic, dry up and drop or fall off (Agrios 1988). *A. cassiae* is mainly associated with the following two diseases:

1. Leaf spot of *Cassia* sp.

Alternaria cassiae causes extensive necrosis of epicotyls, hypocotyls, cotyledons, and true leaves of sicklepod seedlings. The disease has been observed both under natural conditions in the field and on inoculated seedlings (Van Dyke & Trigiano 1987). Leaf lesions on both sides of the leaflets are round or elongated, light to dark-brown with slightly raised margins. Lesions are initially 0.5-2.0mm in diameter, enlarging to 8-10mm and irregularly zonate, covered with tufts of conidiophores (Simmons 1982; David 1991).

2. Leaf spot of *Vigna unguiculata*

According to La Grange & Aveling (1998), foliar symptoms begin as semi-circular, water-soaked lesions at the leaf margins. Lesions enlarge towards the centre of the

leaf, eventually becoming necrotic. Sporulation is visible with the naked eye on the leaf surface as a black velvet mass. Occasionally circular lesions are observed in the centre of the leaf. Lesions begin as small brown spots, surrounded by a yellow chlorotic halo. The lesions enlarge and become water-soaked and a black mass of conidia is visible on the brown, necrotic tissue surfaces. Symptoms have been observed in the field and on inoculated seedlings (La Grange & Aveling 1998).

2.2.7 Epidemiology

Alternaria spp. are extremely resistant to adverse environmental conditions, and this has enabled them to thrive under a wide range of climatic conditions (Rotem 1994). Plant pathogenic species of *Alternaria* over-winter as mycelium or spores in/on seeds and as mycelium in infected plant debris (Agrios 1988; Rotem 1994). Survival in nature is most commonly and frequently ensured through the formation of special resting bodies, such as sclerotia or chlamydospores. Chlamydospores have been reported for a few *Alternaria* spp., such as *Alternaria raphani* Groves & Skolko (Atkinson 1953) and *Alternaria brassicae* (Berk.) Sacc. (Rotem 1994). Microsclerotia have also been found in *Alternaria dauci* (Kuhn) Groves & Skolko from carrots (*Daucus carota* L.) (Rotem 1994). This seed-borne fungus may attack seedlings, usually after emergence, and cause collar rot, damping-off or stem lesions. Spores that are produced abundantly, especially during frequent rains and heavy dews, are blown in from mycelia growing on weeds, infected cultivated plants or infested debris (Agrios 1988). Airborne dispersal is the main mechanism for the distribution of inoculum of *Alternaria* spp. (Rotem 1994), although splash dispersal has occasionally been reported (Rangel 1945). The germinating spores penetrate

susceptible tissue directly or through wounds and soon produce conidia. Conidia are detached easily and are then further spread by wind, splashing rain or tools. Diseases caused by *Alternaria* spp. are generally more prevalent on older, senescing tissue. Plants of low vigour, or poor nutrition or those under some kind of physiological stress caused by unfavourable environmental conditions, insects or other diseases are highly susceptible to infections (Agrios 1988).

2.2.8   Control

Alternaria diseases are primarily controlled through the use of resistant varieties, disease free or treated seed and through the use of chemical sprays such as the following fungicides: maneb, mancozeb, chlorothalonil, captafol and fentin hydroxide (Agrios 1988). Fungicides should be applied soon after the seedlings emerge or are transplanted. For effective control, applications must be repeated at 1-2 week intervals, depending on disease prevalence and severity and the frequency of rain (Agrios 1988). Crop rotation and general field sanitation also help to reduce the amount of inoculum for subsequent plantings of susceptible crops. Covering the greenhouse with special UV-light-absorbing film can drastically reduce infections by some *Alternaria* spp. The filtering out of UV-light inhibits spore formation by these fungi (Agrios 1988). In South Africa, captab (captan), mancozeb, and triforine are some of the fungicides generally recommended for the control of Alternaria diseases on flowers, fruits and vegetables (Nel *et al.* 1999). Boelema & Ehlers (1967) and Naude (1988) recommended that onion seed (*Allium cepa* L.) should be treated with thiram to control *Alternaria porri* (Ellis) Ciferri. Furthermore, according to Maude (1966), thiram could eliminate infection of carrot seeds by *A. dauci.*

Maude & Bambridge (1991) found that topical applications of the fungicide iprodione gave effective eradication of internally seed-borne *A. dauci* of carrot. Iprodione was also the most effective in reducing infection of broccoli seed (*Brassica oleracea* L. var. *italica*) with *Alternaria brassicicola* (Schwein.) Wiltsh. (Sivapalan 1993). According to Aveling *et al.* (1993) a hot-water soak (50°C for 20 min) effectively reduced *A. porri* on onion seeds.

2.3   INTERACTION BETWEEN HOST AND PATHOGEN

2.3.1   Interaction between *A. cassiae* and different hosts

In a host/pathogen interaction study of sicklepod and *A. cassiae*, Van Dyke & Trigiano (1987), found that conidia of *A. cassiae* germinated within 2-3h after inoculation. Mims *et al.* (1997), reported conidial germination only after 3h. An average of six germ-tubes per conidium was observed on sicklepod seedlings 18h-post inoculation (hpi) (Van Dyke & Trigiano 1987). According to Mims *et al.* (1997), two modes of conidium germination were observed in *A. cassiae*. Internal germination, the less commonly observed mode, involved the development of germ-tubes that arose from cell sites inside the conidia. An internal germ-tube developed from one cell and typically grew into or through one or more adjacent cells before emerging from the conidium (Mims *et al.* 1997). The more common mode of germination involved the development of germ-tubes from cell sites directly on the conidium surface. Developing germ-tubes initially appeared as slight bulges on the conidium surface. No special germ pore regions were observed in the conidium wall (Mims *et*

al. 1997). Germ-tubes appeared to push their way through the conidium wall. Germ-tube length varied from 50-300μm and branched frequently (Van Dyke & Trigiano 1987). Developing germ-tubes grew out in all directions from conidia. Some grew almost directly down towards the underlying membrane, while others grew upwards, away from the substrate (Mims *et al.* 1997).

Germ-tubes and their branches usually terminated in appressoria and occasionally intercalary appressoria were also observed (Van Dyke & Trigiano 1987). Appressoria either formed directly on epidermal cells or over stomata. Sometimes germ-tubes grew towards stomata, whereas others passed near stomata, but without forming appressoria. Penetration pegs were occasionally seen under appressoria (Van Dyke & Trigiano 1987). According to Mims *et al.* (1997), no evidence of appressorium development by germ-tubes was observed. Van Dyke & Trigiano (1987) reported the formation of amorphous material around germ-tubes and appressoria. Scanning electron microscopy studies by Mims *et al.* (1997) showed that extra-cellular material was associated with conidia and germ-tubes of *A. cassiae*. It was most commonly observed in the immediate vicinity of developing germ-tubes.

Van Dyke & Trigiano (1987) reported that cotyledon tissue of sicklepod became necrotic by 18hpi. Where appressoria had formed over stomata, guard cells were necrotic and when appressoria formed directly on epidermal cells, mesophyll cells beneath the appressoria were also necrotic (Van Dyke & Trigiano 1987). Hyphal penetrations were rarely observed prior to necrosis of mesophyll cells, but many mesophyll cells located beneath appressoria were necrotic before penetration. The death of cells in advance of fungal penetration, suggests that a diffusible toxin might

be produced by the pathogen (Van Dyke & Trigiano 1987). Numerous studies on a wide variety of pathogenic *Alternaria* spp., have shown that these fungi produce toxins and that they are responsible for much of the disease symptoms on the host (Brian *et al.* 1952; Gilchrist & Grogan 1976; Yoder 1980). By 66hpi collapsed palisade and spongy mesophyll cells were evident in sicklepod plants infected by *A. cassiae* (Van Dyke & Trigiano 1987). Furthermore, according to Van Dyke & Trigiano (1987), the formation of cambium was observed in the host tissue and the number of cells was greater in the lesion area than in the adjacent healthy areas. These authors suggested that a diffusible fungal compound caused the host cells to respond by initiating cell division and that cambium formation and hyperplasia may possibly represent host-defence responses.

2.3.2   Interaction of other *Alternaria* spp. and their hosts

Angell (1929) reported that germination of conidia of *A. porri* on onion leaves occurred within 3h and Fahim & El-Shehedi (1966) found that a majority of *A. porri* conidia germinated within 24h. Everts & Lacy (1996) reported that 90% of *A. porri* conidia germinated on the onion leaf surface after 24h of dew and Aveling *et al.* (1994) found that 96% of conidia of *A. porri* germinated within 24hpi. Conidia of *A. porri* germinated from more than one cell and germ-tubes grew in any direction across the leaf surface usually terminating in appressoria over stomata and on epidermal cells (Fahim & El-Shehedi 1966; Everts & Lacy 1987; Aveling *et al.* 1994). Nolla (1927) and Angell (1929) reported that *A. porri* penetrated via stomata of onion leaves and that no direct penetration through epidermal cells occurred. Walker (1921) reported penetration through stomata and through wounds in the epidermis.

However, evidence of penetration occurring through stomata and directly through unwounded epidermal cells were observed by numerous authors (Fahim & El-Shehedi 1966; Sherf & MacNab 1986; Everts & Lacy 1987; Aveling *et al.* 1994). Changsri & Weber (1963) reported that *A. brassicae* (Berk.) Sacc. penetrated crucifers directly or through stomata. A host-pathogen interaction study between *A. tenuis* Nees and *Phaseolus vulgaris* L. conducted by Saad & Hagedorn (1969) revealed that the pathogen made numerous penetrations through stomata and developed primary hyphae in the substomatal cavities by 24hpi. Direct penetration was less common and usually occurred after 24h.

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CHAPTER 3

A NEW FOLIAR DISEASE OF COWPEA CAUSED BY *ALTERNARIA CASSIAE*

3.1   Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is an important, indigenous food and fodder crop in Africa. In South Africa small-scale farmers in areas with low agricultural potential particularly grow the crop (Quass 1995). Cowpeas are susceptible to a wide range of pests and pathogens that attack the crop at all growth stages (Allen 1983). Diseases may even pose a more serious threat when cowpea production is enhanced and the crop is grown on a larger scale and in monoculture. A literature survey revealed that there are some 40 species of fungi that are known to be pathogens of cowpea. Seven of these pathogens are the causal agents of foliar diseases on cowpea in South Africa.

- *Ascochyta pisi* Lib.   (Doidge 1924)

- *Cercospora cruenta* Sacc.   (Doidge 1924)

- *Colletotrichum dematium* (Pers.) Grove   (Smith & Aveling 1997)

- *C. lindemuthianum* Sacc. & Magnus   (Doidge & Bottomley 1931)

- *Septoria vignae* P. Henn.   (Gorter 1977)

- *Rhizoctonia solani* Kühn   (Doidge 1924)

- *Uromyces appendiculatus* Barl.   (Doidge *et al.* 1953)

General field surveys of various cowpea-growing areas in Botswana and South Africa were conducted. A particularly destructive foliar disease was noted in the warm regions. Unusual high rainfalls were recorded during the growing season and seem to aggravate the expression of the disease. The aim of this study was to identify and study this disease in greater detail.

3.2 Materials and Methods

3.2.1 Field collection and identification

Cowpea fields were visited in Botswana, and Malekutu (Mpumalanga) and Roodeplaat (Gauteng) in South Africa. Although various fungal diseases were observed, the field identification and collection was focussed on only one disease. The disease was preliminary identified, photographed and samples of the diseased material were collected and kept in cold storage during transportation to the laboratory. Diseased leaf material was surface sterilised in 1% (w/v) sodium hypochlorite for 1min, rinsed three times in sterile distilled water (SDW), cut with a sterilized scalpel into small pieces ($5mm^2$) and plated onto potato dextrose agar (PDA) (Merck) amended with 0.025% chloramphenicol. Single spores were also aseptically transferred with a dissecting needle from diseased plant material onto V8-agar and PDA amended with chloramphenicol. The plates were incubated in a 12hUV-light/12h dark cycle at 25ºC. The pathogen was consistently isolated and preliminary identified as *Alternaria cassiae*, using light microscopy, and compared to similar identifications available in literature. An isolate was identified by the Agricultural Research Council (ARC) - Plant Protection Research Institute, Pretoria, South Africa as *Alternaria cassiae* Juriar & Khan, and was deposited in the National Collection of Fungi, Pretoria, South Africa (PPRI 6393). Diseased plant material was also viewed under the stereo-microscope for the presence of conidia.

3.2.2 Koch's Postulates

3.2.2.1 Fungal cultures

The isolate PPRI 6393 was sub-cultured onto V8-agar and maintained in a 12h UV-light/12h dark regime at 25ºC.

3.2.2.2 Plant material

Cowpea seeds (cultivar Rhino obtained from ARC - Grain Crops Institute, Potchefstroom, South Africa) were planted in plastic pots (15cm^3) filled with pasteurised bark growing-media and maintained in a glasshouse at 25ºC (±1ºC). The leaves of 8-wk-old healthy plants were used for inoculation.

3.2.2.3 Inoculation procedure

Inoculum was prepared aseptically by pouring 2ml sterile distilled water into Petri dishes containing the sporulating culture and agitating it with a glass rod. Cowpea leaves were inoculated by painting them with a spore suspension until run-off. As negative and positive controls, plants were respectively inoculated with sterile water or left uninoculated. The plants were placed in a dew chamber for 48h and then returned to the glasshouse and maintained at 25ºC (±1ºC). Cowpea plants were observed regularly for the development of symptoms. Re-isolation of the fungus was done from all the inoculated plants that developed symptoms. Leaf lesions were excised from the diseased leaf material sterilised and plated out as previously described. Single spores were also aseptically transferred from the diseased leaf material onto V8-agar and PDA amended with chloramphenicol. Plates were

incubated at 25°C in a 12h UV-light/12h dark cycle. Fungal growth and spores were compared with the original isolated culture of *Alternaria cassiae.*

3.2.3   Field screening

In March 1998, *A. cassiae* was observed in cowpea fields at the ARC-Vegetable and Ornamental Plant Institute, Roodeplaat, Pretoria. In the growing season of 1999/2000 a cowpea trial was conducted using a cultivar (IT 82D889) obtained from the International Institute for Tropical Agriculture (IITA), Nigeria, which showed disease symptoms the previous season. Prior to planting the area was fertilized with 1.65kg super phosphate and 1.65kg potassium chloride. The trial was planted in November 1999. Four replications of four rows were planted, with a spacing of 0.1 x 0.5m and a spacing of 2m between each of the four blocks. The trial was established with initial supplement irrigation of 20mm a week. At 6 weeks the supplement irrigation was terminated. The trial was screened for disease symptoms caused by *A. cassiae,* which were first noted early in January 2000. A disease rating scale was developed and the disease severity was evaluated during the last week of January. Twenty plants per replication, and three leaves per plant were chosen randomly to record disease severity within each replications. Plants were scored on a 0 (healthy) to 5 (severely infected) scale (Table 3.1). Fig. 3.1 is an example of the leaves used to compile the disease rating scale. The disease severity data from the three leaves were pooled to obtain an average value for each plant. Disease severity was calculated using the formula of Sherwood & Hagedorn (1958):

Disease severity (%) = Σ (no. of plants in a disease scale category) x (specific disease scale category) / (total no. of plants) x (maximum disease scale category) x 100.

Table 3.1   Disease severity rating scale used to record *A. cassiae* symptoms on cowpea leaves.

Disease severity	Disease symptoms on cowpea leaves
0	No visible expression of the disease
1	Small reddish-brown lesions (1-2mm), surrounded by a yellow halo, in the centre of the leaf or on the leaf margins.
2	Lesions enlarge to ±10mm in diameter and become necrotic.
3	Brown, water-soaked lesions up to 20mm in diameter.
4	Necrotic lesions (>20mm) with black velvet masses of conidia, covers more than 25% of the leaf surface.
5	Leaf lesions > 20mm in diameter. Profuse black masses of conidia. Shot-hole symptoms are present and more than 50% of the leaf is destroyed. The rest of the leaf shows signs of chlorosis.

3.3 Results

3.3.1 Field collection and identification.

Foliar symptoms began as semi-circular water-soaked lesions at the leaf edges. Lesions enlarged towards the centre of the leaf, becoming necrotic. Sporulation was visible with the naked eye on the leaf surface as a black velvet mass (Fig. 3.2). Occasionally circular lesions were also observed in the centre of the leaf (Fig. 3.3). Lesions began as small brown to reddish brown spots, surrounded by a yellow halo. Lesions enlarged and became water-soaked, and black masses of conidia were visible on the brown necrotic tissue surface.

During cowpea field surveys at Roodeplaat, Pretoria in March 1999 and 2000, black velvet masses of conidia were also observed on the stems and pods (Fig. 3.4) of cowpea plants. The conidia population was a mixture of long beaked conidia of *A. cassiae* and conidial chains (> 4 conidia) of *Alternaria alternata* (Fr.) Keissler.

3.3.2 Koch's Postulates.

None of the control plants that were either inoculated with sterile distilled water or left un-inoculated developed disease symptoms. All the cowpea plants inoculated with the *A. cassiae* spore suspension, developed disease symptoms identical to the initial symptoms observed in the field after 7-14 days. *A. cassiae* was re-isolated from these plants and produced cultures identical to those of the original isolate. Characteristic long beaked conidia were also present in these cultures.
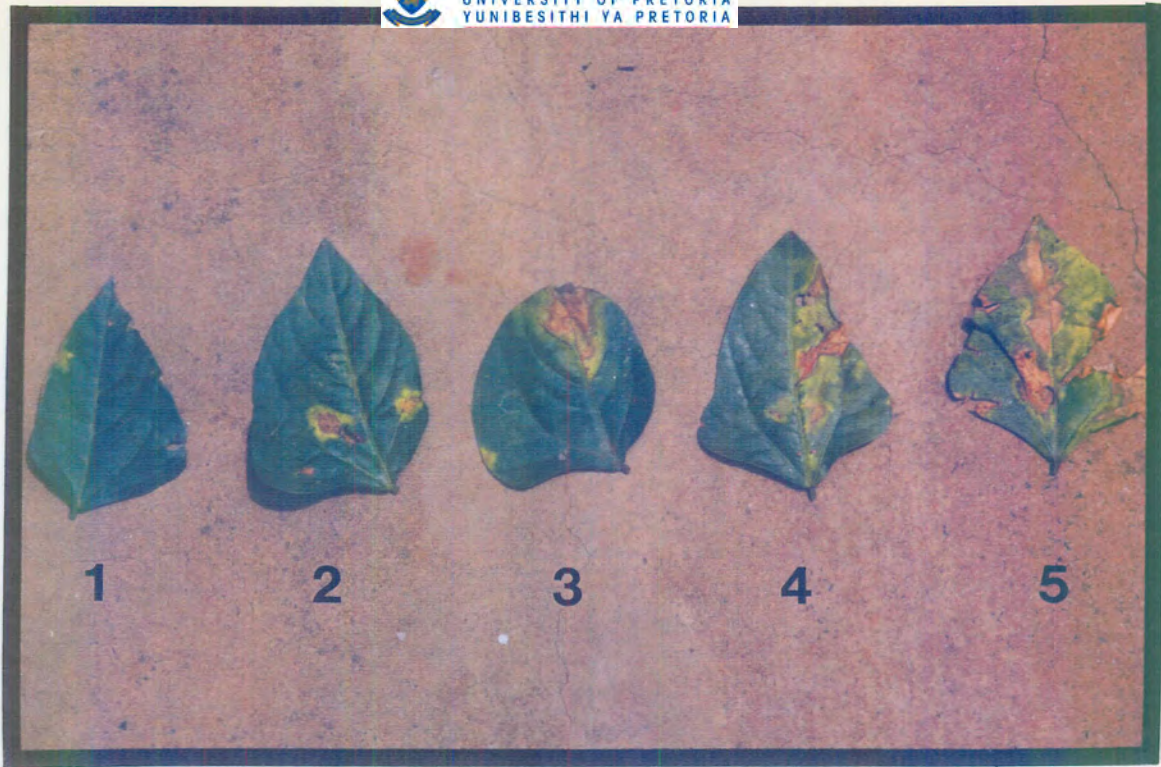
Fig. 3.1   Example of cowpea leaves infected with *A. cassiae* used to compile the disease severity rating scale.

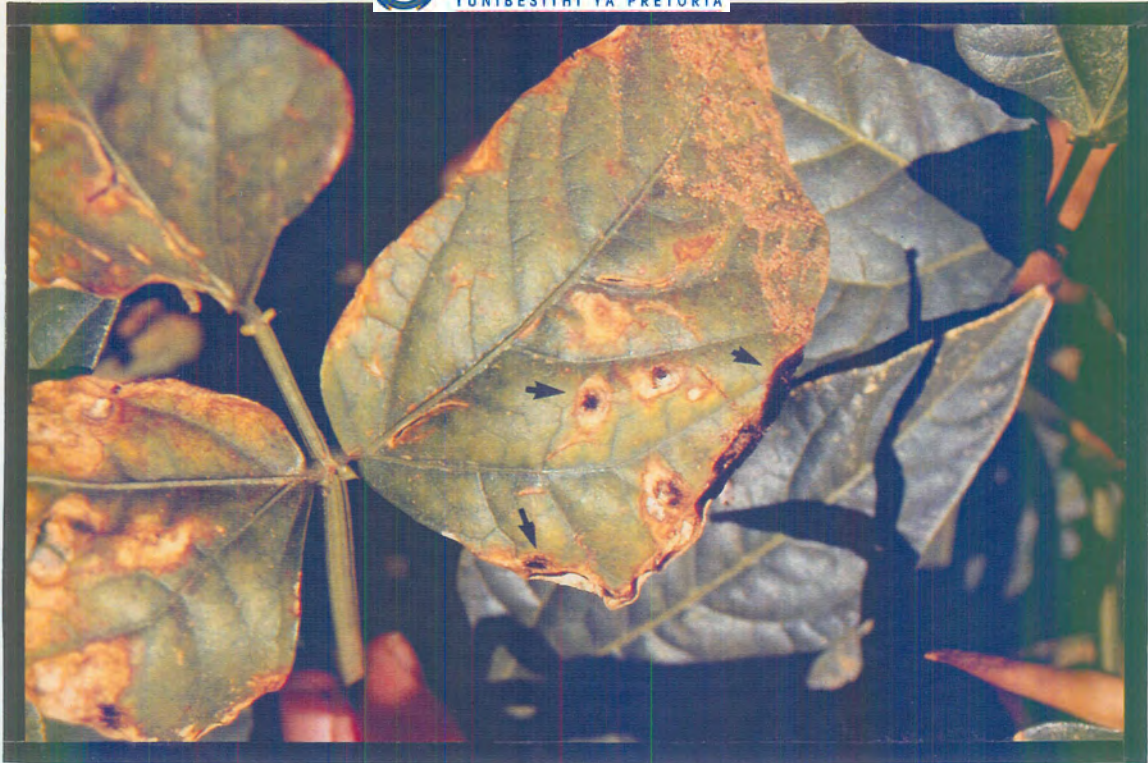**Fig. 3.2**   Disease symptoms on the leaves of cowpea caused by *A. cassiae.*

Fig. 3.3 Circular lesions in the centre of a cowpea leaf with typical black masses of conidia of *A. cassiae*.

Fig. 3.4 Fungal growth and sporulation of *A. cassiae* on pods of cowpea.

3.3.3 Field screening

Leaf symptoms either began at the leaf edges or in the centre of the leaf, as described. Disease development progressed as the plants matured, and pods were set. The severity of *A. cassiae* in the trial was measured in terms of percentage disease severity for each of the four replications (Table 3.2).

Table 3.2   Percentage disease severity of *A. cassiae* on 4 replicates of cowpeas at Roodeplaat, Pretoria.

Percentage disease severity				
Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean
50%	46%	47%	43%	47%

3.4   Discussion

This is the first report of *A. cassiae* on cowpea worldwide. The presence of a disease on any crop has serious implications on the cultivation of that crop. Many aspects of both the pathogen and the host plant are unknown, providing many research opportunities. Cowpeas are increasingly being produced on a larger scale in Southern Africa (Coetzee 1995), and large areas cultivated solely under cowpeas increase the potential threat of diseases.

The disease was generally observed in warmer areas of Southern Africa with high rainfall during the 1998/1999-cowpea growing seasons. Cowpea plants in a field trial, established at Roodeplaat, Gauteng (1999/2000), showed an average disease severity of 47%. Fungal growth of *A. cassiae* was visible on leaves, stems and pods. This had a negative effect on the quality of the crop. Both the leaves and grain are used as vegetables particularly by small subsistence farmers, and the presence of the disease may reduce yield substantially resulting in food shortage.

It is important to rapidly identify the pathogen and disease in order to be able to control and eliminate it before it becomes established in the crop.

This chapter reported on a new disease on cowpea, which poses a serious threat to production. A visual aid for the identification and severity rating of *A. cassiae* on cowpea was also developed.

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Disease Notes

Plant Disease "Disease Notes" are originally published on-line and are available several weeks before being reprinted here. A note's official date of publication is the date it was placed on-line. Information concerning on-line access appears at the front of this journal under *Editorial Policies*.

First Report of *Alternaria cassiae* on Cowpea. N. la Grange, ARC-Roodeplaat P/Bag X293, Pretoria 0001, South Africa, and T. A. S. Aveling, Department of Botany, University of Pretoria, Pretoria 0002, South Africa. Plant Dis. 82:1171, 1998; published on-line as D-1998-0727-01N, 1998. Accepted for publication 23 July 1998.

A destructive foliar disease was observed during field surveys of cowpea (*Vigna unguiculata* (L.) Walp.) in the Mpumalanga and Gauteng provinces in South Africa. Foliar symptoms begin as semicircular water-soaked lesions at the leaf margins. Lesions enlarge toward the center of the leaf, becoming necrotic. Sporulation is visible with the naked eye on the leaf surface as a black velvet mass. Occasionally, circular lesions are observed in the center of the leaf. Lesions begin as small brown spots, surrounded by a yellow halo. The lesions enlarge and become water-soaked, and black masses of conidia are visible on the brown, necrotic tissue surface. *Alternaria cassiae* (A. M. M. Juriar & A. Khan) was consistently isolated from the diseased leaf material. An isolate of the causal pathogen was identified by and deposited with the National Collection of Fungi, Plant Protection Research Institute, Pretoria, South Africa, and designated with the number PPRI 6393. Koch's postulates were proven by inoculating the leaves of cowpea seedlings with a 10^6 conidia ml^{-1} suspension. Inoculated plants were maintained in a humidity chamber for 48 h and then returned to the greenhouse. After approximately 7 days, symptoms resembling those observed in the field were apparent. *A. cassiae* was reisolated from diseased tissue. The same foliar disease was observed on cowpea in Botswana. *A. cassiae* was isolated and identified from the characteristic lesions.

First Report of Cucumber Mosaic Cucumovirus Subgroup 1 in South Africa, from Banana with Infectious Chlorosis. G. Pietersen, M. S. Staples, and G. G. F. Kasdorf, ARC-Plant Protection Research Institute (PPRI), Private Bag X134, Pretoria, 0001; and J. E. Thomas, Queensland Department of Primary Industries, 80 Meiers Road, Indooroopilly, Queensland 4068, Australia. Plant Dis. 82:1171, 1998; published on-line as D-1998-080301-01N, 1998. Accepted for publication 24 July 1998.

Cucumber mosaic cucumovirus (CMV) is the etiological agent of infectious chlorosis disease in bananas (*Musa* spp.). In South Africa, diagnosis of CMV on banana has been based only on symptoms (1). A Grande Naine (Cavendish, AAA) plant with typical infectious chlorosis disease was obtained from Letsitele. Sap from this plant was inoculated to indicator plants. Virus was isolated by two serial local lesion transfers on *Chenopodium quinoa* and maintained on *Nicotiana benthamiana*. It was identified as a subgroup 1 CMV based on its reaction to CMV DTL-, but not CMV ToRS- monoclonal antibodies in double antibody sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA). The identity was further confirmed by the polyhedral particle morphology under the electron microscope by negatively staining, and decoration in immuno-electron microscopy with antiserum to a local tomato isolate of CMV. Infectious chlorosis disease symptoms were induced by sap inoculation to healthy Grande Naine plants, and the virus detected by ELISA. This is the first confirmed diagnosis of CMV on bananas in South Africa. The isolate was deposited in the PPRI Virus Collection.

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Pseudomonas syringae* Leaf Blight, a New Disease of *Kalmia latifolia. M. L. Putnam, Oregon State University, Botany and Plant Pathology, Corvallis 97331-2902. Plant Dis. 82:1171, 1998; published on-line as D-1998-0811-01N, 1998. Accepted for publication 6 August 1998.

In spring and fall of 1997, and in February 1998, *Kalmia latifolia* cv. Olympic Fire plants with severe leaf blight symptoms were submitted to the Oregon State University Plant Clinic from a commercial nursery. The primary symptom was a dark purple leaf blight, often associated with the leaf mid-rib. Disease progressed down the petioles and into twigs, causing blackening of affected tissues and leaf drop. Abundant bacterial streaming was observed oozing from the margins of affected tissue when examined at ×100. Isolations from affected tissues were made onto King's medium B (KB). A fluorescent bacterium was recovered and identified as *Pseudomonas syringae* by the Biolog system of identification. Identity was confirmed by fatty acid methyl ester analysis performed by Larry Barnes (Texas A&M University, College Station). Attempts to determine the pathovar were unsuccessful. A single colony isolate of the bacterium was raised on KB. Koch's postulates were completed by the following procedures. A bacterial suspension was made from a 24-h-old agar culture of this isolate with phosphate buffer with 0.2% gelatin (PBG). The concentration of the suspension was adjusted to 8×10^7 cells per ml by direct enumeration. Five milliliters of the suspension was atomized onto young leaves on six twigs of *Kalmia latifolia*. Controls consisted of young leaves on four twigs atomized with 5 ml of PBG. Twigs receiving the inoculum or the PBG were enclosed in plastic bags and maintained at room temperature near a north-facing window. Symptoms appeared 6 days later: dark purple spots on the margins of inoculated leaves and blight symptoms near the leaf mid-rib. Symptoms did not appear on PBG-sprayed leaves. *Pseudomonas syringae* was successfully reisolated from surface-disinfested inoculated leaves but not from leaves sprayed with PBG. This is the first report of *Pseudomonas syringae* causing a leaf blight of *Kalmia*.

First Report of the Cymbidium Mosaic Potexvirus (CymMV) Infecting the Terrestrial Orchid *Phaius tankervilliae* in Costa Rica. L. Moreira and W. Villalobos, Centro de Investigacíon en Biología Celular y Molecular (CIBCM), Universidad de Costa Rica; H. T. Hsu, Florist & Nursery Plants Research Unit, USDA-ARS, Beltsville, MD 20705-2350; E. Rodríguez-Cerezo, Centro Nacional Biotecnología (CNB CSIC), Madrid; and C. Rivera, CIBCM, Universidad de Costa Rica. Plant Dis. 82:1171, 1998; published on-line as D-1998-0812-01N, 1998. Accepted 29 July 1998.

In 1996, plants of the terrestrial orchid *Phaius tankervilliae* from a nursery in the Central Valley of Costa Rica were observed with mild to severe foliar symptoms of chlorotic streak. No differences were observed in growth, bulb production, flowers, or flowering time between symptomatic and asymptomatic plants, except the symptomatic plants had earlier senescence. Occasionally, the flowers displayed symptoms of chlorosis and white rings in the sepals. Extracts from symptomatic leaves were concentrated by differential centrifugation and analyzed after sucrose gradients. Negative staining of fractions from gradients from symptomatic plants showed the presence of filamentous viral particles 500 by 17 nm. Purified particles contained a single major protein of about 28 kDa as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a single RNA of about 7 kb, which is greater than the 6.2 kb reported (GenBank). These data suggest the presence of a potexvirus in symptomatic plants (1,2). In enzyme-linked immunosorbent assays, symptomatic plants reacted strongly with antiserum specific for Cymbidium mosaic potexvirus (CymMV). This is the first report of CymMV in *P. tankervilliae* in Costa Rica.

References: (1) J. A. Frowd and J. H. Tremaine. Phytopathology 67:43, 1977. (2) H. T. Hsu et al. Phytopathology 82:491, 1992.

CHAPTER 4

THE EFFECT OF CULTURE MEDIA AND OTHER FACTORS ON THE GROWTH AND SPORULATION OF *ALTERNARIA CASSIAE*

The effect of culture media and other factors on the growth and sporulation of *Alternaria cassiae*

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Abstract

Alternaria cassiae Juriar & Khan is the causal agent of a new foliar disease on cowpea (*Vigna unguiculata* (L.) Walp). However, in culture the pathogen sporulated inconsistently and in order to optimise sporulation for further studies the following *in vitro* conditions were studied: six culture media, temperatures ranging from 5-40°C, three light treatments and the effect of wounding the cultures. *A. cassiae* grew well and abundantly produced spores when maintained on V8-agar at 25°C in a 12h UV-light/12h dark cycle.

Keywords: *Alternaria cassiae*, cowpea, sporulation, wounding

Alternaria cassiae Juriar & Khan was first isolated and identified as the causal pathogen of a new foliar disease of cowpea (*Vigna unguiculata* (L.) Walp) in 1998 (La Grange & Aveling 1998). It was soon evident that cultures of *A. cassiae* maintained on potato dextrose agar (PDA), produced few, if any, spores at inconsistent intervals. Simmons (1982) reported that *A. cassiae* isolated from *Cassia* spp. grew readily on V8-agar and sporulated abundantly on V8-agar held in a light/dark cycle. However, preliminary studies indicated that *A. cassiae* isolated from cowpea did not sporulate well under these light conditions. It is well known that many *Alternaria* spp. do not sporulate consistently well under *in vitro* conditions (Rotem 1994). In view of the reliance on high spore concentrations for disease development and control strategy studies, the following investigation was done to obtain suitable *in vitro* conditions for the constant production of large numbers of spores.

An isolate of *A. cassiae* (PPRI 6393) was obtained from the National Collection of Fungi, Pretoria, South Africa. Stock cultures of the fungus were maintained on V8-agar and incubated at 25°C under 24h UV-light. The following factors were investigated; six different culture media, temperature, three light treatments and wounding of the cultures. Four Petri dishes containing medium were used for each treatment in each experiment. Each plate was inoculated in the centre with an inverted 7mm diameter disc from the advancing margins of actively growing 12d old fungal colonies. The following culture media were prepared aseptically to determine the optimal media for growth and sporulation of *A. cassiae*: Potato dextrose agar (PDA) (Merck), malt extract agar (MEA) (Merck), water agar (WA) (Merck), cornmeal agar (CMA) (Merck), Czapek dox (CDA) (Oxoid) and V8-agar (V8-juice;

Campbell and agar; Merck). Plates were incubated at 25°C in a 12h UV-light/12h dark regime. Colony growth was assessed on day six by measuring two diameters (in mm) of colonies at right angles to each other. The first day of visible sporulation was recorded, and on day 12, three replicates of each media and three samples per replicate were used to determine the spore concentration. One millilitre of sterile distilled water was poured into each Petri dish. The culture was agitated with a sterile glass rod and the spore suspension was transferred to a Kova Glasstic Slide (haemocytometer) and spores μl^{-1} was determined. On day 8 the fungal cultures on the different media were described using the Mycological Colour Chart designed by Rayner (1970). To determine the optimal temperature for mycelial growth V8-agar plates containing agar discs of *A. cassiae* were placed at 5, 10, 20, 25, 30, 35 and 40°C in 24h dark. Colony diameter was measured on day 6. V8-agar plates inoculated with the fungus were incubated at 25°C and placed in three light regimes; 24h dark, 24h UV-light and, 12h UV-light/12h dark. Growth and sporulation were measured on day 6 and 12, respectively. The effect of wounding cultures was determined on the six different culture media studied. Plates were incubated at 25°C for 5 days in a 12h UV-light/12h dark cycle. On day six mycelium of each culture was either left intact or wounded by scraping, using a sterile dissecting needle. Plates were re-incubated under the same conditions and spore concentrations were determined on day 9. Data was analysed using the Student T-test ($P = 0.05$).

There were significant differences in the growth (Fig. 1) and sporulation (Table 1) of *A. cassiae* on the six different media. *A. cassiae* grew best on V8-agar (Fig. 1) and produced significantly more spores (Table 1) than cultures maintained on any other media. Within six days, the culture on V8-agar had grown 67.8mm. Colonies were

dark mouse grey to black, with slightly fluffy whitish to pale mouse grey aerial mycelial growth. On CDA, the fungal growth was significantly faster than all the other media, except V8-agar. The colonies were olivaceous grey, covered with a fluffy concentrically ringed creamy and rosy buff mycelial growth. Very sparse white mycelial growth was characteristic of the cultures on WA. Olivaceous grey colonies on PDA were covered with fluffy concentrically ringed cream and rosy-buff sectors. Fungal growth on MEA was significantly slower than on all the other media except CMA. The colonies were fluffy, olivaceous to iron grey and covered with a slightly fluffy layer of white to pale mouse grey aerial mycelia. Colonies on CMA were sparse and white with concentrically ringed zones of darker mycelia. Fungal growth on this media was significantly slower than on all the other media. By day 6, the culture had only grown 33.7mm. Cultures on both CMA and WA showed the first visible signs of sporulation after six days. On day 12, cultures maintained on CDA, had produced a few spores, visible under the dissecting microscope. However, no spores were observed when counted with the haemocytometer. Cultures on PDA produced 6.67 spores μl^{-1} after 12 days. After 14 days, cultures maintained on MEA still produced no spores. According to Rotem (1994) all *Alternaria* spp. sporulate better on media poor in sugars such as V8-juice. Even though mycelial growth of cultures maintained on CDA was high, sporulation was poor. Similar results were found for *Alternaria porri* (Ellis) Cif.. Gupta *et al.* (1987) found that CDA proved best for growth of *A. porri*, but none of the isolates sporulated. Since V8-agar proved to be best for the growth and sporulation of *A. cassiae*, it was used in further experiments.

A. cassiae grew best at 20°C and 25°C on V8-agar when compared with growth at other temperatures (Table 2). No fungal growth was recorded at 5°C and 40°C. At 10°C, a slight fungal growth of 0.75mm was observed. The fungal growth rate

dropped sharply from 20°C to 10°C and from 30°C to 35°C. It appears that temperatures lower and higher than 10°C and 40°C, respectively are the minimum and maximum lethal temperatures for mycelial growth of *A. cassiae in vitro*. According to Angell (1929), the optimal temperature for mycelial growth of *A. porri* was found to be between 22°C and 30°C.

A. cassiae grew best under 24h UV-light (70.25mm after 6 days) on V8-agar (Table 3). Cultures maintained on V8-agar at 25°C in a 12h UV-light/12h dark cycle, grew well, but slightly slower than cultures kept at 24h UV-light. However, sporulation was significantly better in cultures kept in the diurnal (12h UV-light/12h dark) cycle. According to Rotem (1994) irradiation with UV-light is the main element in most formulas for sporulation. In this study, even though cultures maintained under 24h UV-light grew best, they sporulated least. However, the abundant presence of conidiophores indicates that UV-light stimulated the development of conidiophores, but may have inhibited the formation of conidia. The presence of large amounts of conidia present on cultures kept in a 12h UV-light/12h dark regime indicates that conidium formation is induced and favoured by darkness. These results correlate with the statements made by Leach (1967) who reported two phases of sporulation. The first phase leads to conidiophore formation and is stimulated by near ultra-violet; while the second phase of conidium formation proceeds best in darkness and is often inhibited by light.

In general, wounding of the colonies enhanced spore production on the different culture media (Table 4). Sporulation occurred on non-wounded cultures of all the media except MEA and PDA (Table 4). With the exception of PDA, all wounded cultures produced significantly more spores than the non-wounded cultures. Wounded cultures on V8-agar produced approximately 2.5X more spores than the

non-wounded cultures. These results are similar to the findings of Simmons (1982) who reported that sporulation of *A. cassiae* was particularly abundant after scarification of surface mycelium and that undisturbed colonies of dark, sometimes brown-black mycelium tend to develop an overlay of pale grey non-sporulating hyphae. According to Changsri & Weber (1963) cutting and scraping of mycelium stimulated sporulation of *Alternaria brassicae* (Berkeley) Saccardo, *Alternaria brassicicola* (Schweinitz) Wiltshire, and *Alternaria raphani* Groves & Skolko. It was shown in this study that cultures of *A. cassiae* maintained on V8-agar at 25ºC in a 12h UV-light/12h dark cycle sporulated best and that wounding these cultures further increased sporulation.

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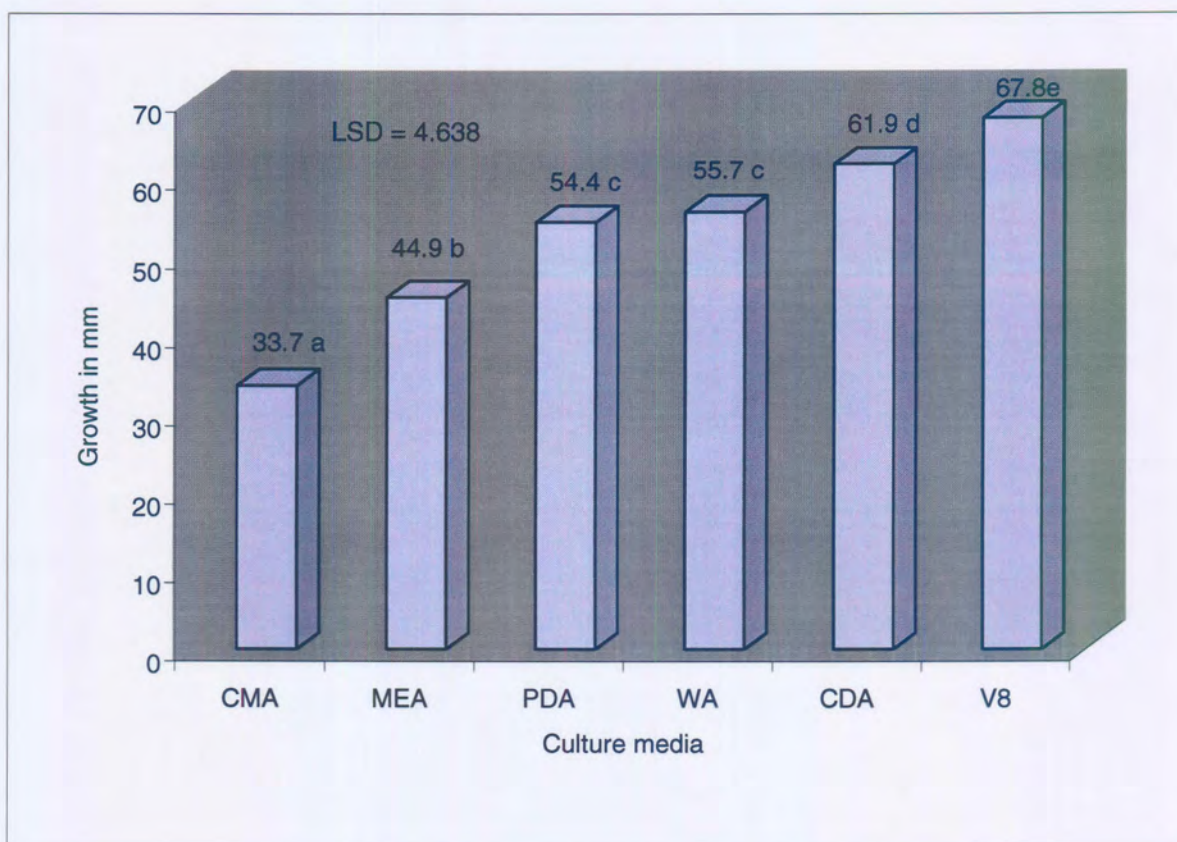
Fig. 1   **Fungal growth of *A. cassiae* after six days on different artificial media. CMA = cornmeal agar, MEA = malt extract agar, PDA = potato dextrose agar, WA = water agar, CDA = Czapeck dox agar and V8 = V8-juice agar**

Table 1. **Spore concentration of *A. cassiae* on six different culture media.**

Media	First day of visible spore formation	Spore concentration (spores μl^{-1}) (On Day 12)
V8-agar	8	45.6 *c
CDA	12	0 a
CMA	6	30.7 b
MEA	None by 14 days	0 a
PDA	12	6.67 a
WA	6	3.7 a

* Each value is the mean of four replications. Values within the column, not followed by the same letter are significantly different ($P = 0.05$), according to the Student T-test.

LSD = 8.323

Table 2. Effect of different
growth of *A. cassiae* after six days on V8-agar in 24h dark.

Temperature range (ºC)	Growth in mm
5	0 *a
10	0.75 a
20	17.63 d
25	17.88 d
30	16.5 c
35	6.63 b
40	0 a

* Each value is the mean of four replications. Values within the column, not followed by the same letter are significantly different (*P*=0.05), according to the Student T-test.

LSD = 0.8159

Table 3. **Effect of three light regimes on the growth and sporulation of** *A.*

cassiae **on V8-agar.**

	Mycelial growth (mm)	Spore concentration (spores μl^{-1})
24h UV-light	70.25 *c	0.6 a
12hUV/12h dark	66.00 b	55.6 b
24h dark	39.25 a	5.2 a
LSD	3.590	12.02

* Each value is the mean of four replications. Values within the column, not followed by the same letter are significantly different (*P* = 0.05), according to the Student T-test.

Table 4. **Effect of wounding on sporulation of** *A. cassiae* **on different culture media after nine days.**

		Wounded	Non-wounded
	LSD	Spore concentration (spores μl^{-1})	Spore concentration (spores μl^{-1})
V8	13.44	60.00* b	25.29 a
CDA	3.231	2.89 a	0.66 a
CMA	5.464	14.22 b	6.22 a
MEA	2.584	3.33 b	0 a
PDA	0	0 a	0 a
WA	4.404	7.78 b	1.32 a

* Each value is the mean of four replications. Values within a specific row allocated to one media treatment, not followed by the same letter are significantly different ($P = 0.05$), according to the Student T-test.

CHAPTER 5

INFECTION STUDIES OF

ALTERNARIA CASSIAE ON

COWPEA

Infection studies of *Alternaria cassiae* on cowpea.

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Abstract: Cowpea leaves and stems were inoculated with conidia of *Alternaria cassiae*. Conidial germination, appressorial formation, penetration and colonization of the plant surface were studied. Multiple germ-tubes developed randomly from each conidium and grew in any direction across the leaf surface. Terminal (71%) or intercalary (29%) appressoria were formed above epidermal cells or over stomata. Penetration of the plant surface, whether directly through the epidermis or via stomata occurred with or without the formation of appressoria. Following penetration through the stoma, bulbous primary hyphae developed within the sub-stomatal cavity. Secondary hyphae developed from the primary hyphae and grew within the intercellular spaces penetrating epidermal and mesophyll cells. Conidiophores emerged directly through the epidermis or through stomata.

Introduction

Alternaria cassiae Juriar & Khan, is the causal agent of a new foliar disease on cowpea (*Vigna unguiculata* (L.) Walp) (La Grange and Aveling 1998). Leaf symptoms begin as semi-circular water-soaked lesions at the leaf margins. Lesions enlarge towards the centre of the leaf becoming necrotic. Sporulation is visible with the naked eye on the leaf surface as a black velvet mass. Circular lesions are occasionally observed in the centre of the leaf. These lesions developed differently from those at the leaf margins, beginning as small brown spots, surrounded by a yellow halo. Lesions enlarge and become water-soaked, and black masses of conidia are visible on the brown, necrotic tissue surface (La Grange and Aveling 1998).

Van Dyke and Trigiano (1987) studied the host/pathogen interaction of *A. cassiae* on sicklepod (*Cassia* sp.) using light microscopy and scanning electron microscopy. Little research is however available on the host/pathogen interaction of cowpea and different fungal pathogens, and no studies have been done on the infection of cowpea by *A. cassiae*. The objective of this investigation was to study the pre-penetration, penetration and the infection process of cowpea by *A. cassiae* using light and scanning electron microscopy.

Materials and methods

Fungal cultures

An isolate of *A. cassiae* (PPRI 6393) was obtained from the National Collection of Fungi, Pretoria, South Africa. *A. cassiae* was sub-cultured on cornmeal agar (CMA) and V8-Juice agar (V8), and incubated at 25°C in a 12 h UV-light/12 h dark cycle to enhance sporulation.

Plant material

Cowpea seeds (cv. Rhino) (four per pot) were planted in plastic pots (15 cm^3) containing pasteurised soil and maintained in a greenhouse at 25°C (±1ºC). Plants were watered daily. The stems and leaves of 6-8 week old seedlings were used for infection studies.

Inoculation procedure

Inoculum was prepared aseptically by pouring 2 ml SDW into Petri dishes containing a 14 d old sporulating culture and agitating it with a glass rod. Cowpea stems and leaves were inoculated by painting them to run-off with a 1x10^5 conidia ml^{-1} suspension of *A. cassiae* spores. Inoculated plants were placed in a mist chamber and kept at 25°C (±1ºC) until sampling.

Light microscopy

To study the infection process and quantify the percentage of pre-penetration structures stems and leaves were cut into 30 mm and 5 mm^2 pieces, respectively at various time intervals after inoculation. The epidermis of specimens were stripped, mounted on microscope slides and stained with lactophenol blue. Micrographs were made with a Nikon Optiphot and a Zeiss photomicroscope.

Scanning electron microscopy (SEM)

To study pre-penetration and infection behaviour, 5 mm stem segments (sliced in half) and 5 mm^2 leaf pieces were cut from the inoculated plants at various time intervals after inoculation. Plant material was fixed in 2.5% (v/v) gluteraldehyde in

0.075 M phosphate buffer (pH 7.4-7.6). Material was rinsed in the same buffer and then post-fixed in 0.25% (w/v) aqueous osmium tetroxide for 2-4 h. This process was followed by three successive washing steps in distilled water. Material was dehydrated in an ascending ethanol series, critical point dried in a Bio-rad critical point dryer and mounted on stubs. In order to study the infection process beneath the epidermis and within the stems and leaves, the epidermis of some of the specimens was removed using the double-sided tape method of Hughes and Rijkenberg (1985). All specimens were coated with gold in a Polaron sputter coater and examined with a Jeol JSM 840 scanning electron microscope at 5 kV.

Results

Conidia of *A. cassiae* were large and septate, with long filiform beaks (Fig. 1), ranging from 75-200 μm *in vivo*. Germinated and ungerminated conidia adhered strongly to the stem and leaf surfaces, since they were not dislodged during SEM preparation. Extra-cellular amorphous material was observed around conidia and in the immediate vicinity of germ-tubes and appressoria (Fig. 2) on the host surface. Conidia of *A. cassiae* germinated within 2 h of inoculation, and by 3 h post inoculation (hpi), 86% of the conidia had germinated. Each conidium produced several germ-tubes at random positions on the conidium and grew profusely in any direction across the surface (Fig. 3). Occasionally a germ-tube was observed developing from the tip of a filiform beak (Fig. 3). An average of four germ-tubes per conidium was observed, with the most being eight (Fig. 3). Germ-tubes were variable in length (10-250 μm) and branched infrequently (Fig. 4). Eight hours after inoculation the first appressoria were observed (Fig. 2 & 4). Germ-tubes and their

branches usually terminated in bulbous appressoria (71%) (Fig. 4), but intercalary appressoria (29%) were also occasionally observed (Fig. 5). Appressoria formed directly on the epidermal cells (74%) (Fig. 4) or on stomata (26%) (Fig. 6). Some germ-tubes grew towards and entered a stoma without forming an appressorium over the stoma (Fig. 7). Other germ-tubes passed near stomata without appressorium formation and showing no direction towards the stomata (Fig. 4). Stomata passed in this way were found in both open and closed states (Fig. 4). Occasionally extensive growth of germ-tubes formed a hyphal network on the host tissue. By 72 hpi, after removing the epidermis, hyphae were seen penetrating the leaf surface either through stomata (Fig. 8) or directly through the epidermis (Fig. 9). Following penetration of the epidermis, bulbous primary hyphae developed below the stoma within the stomatal cavity (Fig. 10). At 96 hpi hyphae were occasionally observed in the mesophyll cells (Fig. 11). Secondary hyphae developed from the primary hyphae and grew intra- and intercellularly passing through adjacent epidermal cell walls (Fig. 12), parallel to the cuticle. Epidermal cells in the immediate vicinity of fungal structures stained dark blue when compared to other epidermal cells not in close proximity of fungal structures (Fig. 13). By 168 hpi, hyphae branched and grew extensively through the mesophyll and epidermal cells (Fig. 14). The first conidiophores emerging through stomata (Fig. 15) or directly through the epidermis (Fig. 16) were only observed at 96 hpi. At 288 hpi black masses of conidia were visible with the naked eye on the necrotic host surface.

Discussion

The results of the infection process of *A. cassiae* on cowpea are generally consistent with that of *A. cassiae* on sicklepod (*Cassia obtusifolia* L.) (Van Dyke and Trigiano 1987) as well as other *Alternaria* spp. on a range of hosts (Angell 1929; Allen, Brown and Kochman 1983; Aveling, Snyman and Rijkenberg 1994; Rotem 1994). Despite taxonomic and pathogenic differences, various *Alternaria* species cause similar patterns of infection (Rotem 1994). Our results confirm those of Van Dyke and Trigiano (1987) and Mims, Rogers and Van Dyke (1997), who reported that conidia of *A. cassiae* germinated within 2-3 hpi, producing multiple germ-tubes that grew in any direction across the leaf surface. According to Angell (1929), conidia of *Alternaria porri* (Ellis) Cif. on onions (*Allium cepa* L.) germinated within 3 h. Rotem (1994) reported that spores of all *Alternaria* species germinate in a remarkably short time and produce one to several germ-tubes. The amorphous extra-cellular material associated with conidia, germ-tubes and appressoria of *A. cassiae* on sicklepod (Van Dyke and Trigiano 1987; Mims et al. 1997) was also present in this study. Previous SEM studies have shown that extra-cellular material is also associated with germ-tubes and appressoria of *Alternaria helianthi* (Hansf.) Tub. & Nish. on sunflower (*Helianthus annuus* L.) (Allen et al. 1983) and *A. porri* on onions (Aveling et al. 1994), and may have an adhesive function. This study found that germ-tubes and their growth were extremely variable, but this is not unusual for *Alternaria* spp. and similar responses have been reported for *A. tenuis* Nees on beans (*Phaseolus vulgaris* L.) (Saad and Hagedorn 1969) and *A. cassiae* on sicklepod (Van Dyke and Trigiano 1987). Van Dyke and Trigiano (1987) reported that germ-tubes of *A. cassiae* and their branches terminated in appressoria, and that intercalary appressoria were also

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occasionally observed. These authors further reported that appressoria formed directly on epidermal cells or over stomata, with about equal frequency. Our results also show the formation of both terminal and intercalary appressoria directly on epidermal cells or over stomata, but the formation of appressoria on epidermal cells was more frequent. In this study germ-tubes occasionally entered through stomata with no appressorium formation, as reported for *A. helianthi* on sunflower (Allen et al. 1983). Results of this study showed both direct and indirect penetration with or without the formation of appressoria, indicating that appressoria are not necessarily obligatory for infection. According to Van Dyke and Trigiano (1987), C.G. van Dyke (unpublished data) similarly found that *Alternaria alternata* (Fr.) Keissler entered its specific host through stomata and by direct penetration with or without appressoria. Von Ramm (1962) also reported that *Alternaria longipes* (Ell. & Ev.) Mason penetrated tobacco (*Nicotiana* sp.) leaves without appressorium formation. *A. cassiae* can be considered a more virulent species due to both direct and indirect modes of penetration. In less virulent *Alternaria* spp. the infection target is limited to wounds and stomata (Rotem 1994). No evidence of specific orientation or long-distance attraction towards stomata was observed and germ-tubes often passed stomata with no apparent trophic response. Stomatal penetration appeared to occur by chance. These results correlated with those of *A. longipes* on tobacco (Von Ramm 1962), *A. cassiae* on sicklepod (Van Dyke and Trigiano 1987) and of *A. porri* on onions (Aveling et al. 1994). The mode of penetration, whether mechanical or chemical was not determined in this study. The presence of infection hyphae growing within the host tissue was first evident after 72 hpi. This is later than the results obtained for *A. porri* on onion (Everts and Lacy 1996), where infection hyphae were observed within the host at 6 hpi. However, these results support the findings of Van Dyke and Trigiano (1987).

They reported that the cells beneath appressoria were not penetrated 18 hpi although they were already necrotic and that intercellular hyphae were only found inside the epidermal cells of sicklepod 66 hpi. As reported for *A. cassiae* on sicklepod (Van Dyke and Trigiano 1987), darkened areas representing discoloured cells as a result of the fungus were also observed in the near vicinity of fungal structures of *A. cassiae* on cowpea. The presence of these discoloured cells indicate that the cells have been disrupted. Van Dyke and Trigiano (1987) reported that as many as 10 cells in the sub-stomatal area beneath appressoria were necrotic, with no evidence of fungal invasion in the tissue. These authors further stated that hyphal penetrations were seldom observed prior to necrosis of mesophyll cells and that the death of these cells in advance of fungal penetration suggests the action of diffusible toxins. Walker and Riley (1982) and Walker and Boyette (1985) have also reported that *A. cassiae* produces a toxin when infecting sicklepod. Development of necrosis in advance of penetration is also associated with other necrotrophic fungi, such as *Stemphylium botryosum* Wallr. f. sp. *lycopersici* on tomato (Rotem 1994). The formation of primary bulbous hyphae within the host has also been reported for *A. porri* on onion (Fahim and El-Shededi 1966; Aveling et al. 1994) and *A. tenuis* on bean (Saad and Hagedorn 1969). In this study secondary hyphae developed from primary hyphae and grew within the intercellular spaces, penetrated and grew intracellular within the epidermal and mesophyll cells. These findings correlate with the results obtained for *A. cassiae* on sicklepod (Van Dyke and Trigiano 1987). These authors reported that intra- and intercellular hyphae were found inside the epidermis and among palisade mesophyll. *A. porri* on onions also grew both inter-and intracellularly (Angell 1929; Aveling et al. 1994). *A. cassiae* grew profusely and by 168 hpi, the pathogen had colonised the host tissue. Leaves were necrotic and the first conidiophores emerging

through the epidermis and stomata were observed, indicating the initiation of conidia formation. Well-developed leaf lesions caused by *A. cassiae* on sicklepod were visible within 66 hpi (Van Dyke and Trigiano 1987).

Much is known about the pre-penetration structures and infection process of other *Alternaria* spp. on their specific hosts. However this is the first study of the infection process of *A. cassiae* on cowpea. This study provides new and relevant information, which will broaden the knowledge of the pre-penetration structures, penetration and colonization of *Alternaria* spp., especially on legumes.

Acknowledgements

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**Legends to figures.**

Figs 1-6. Light and scanning electron micrographs of the conidia and pre-penetration structures formed by *A. cassiae* on cowpea. **Fig. 1.** A mature, beaked conidium of *A. cassiae* with multiple septae. Bar = 10 μm. **Fig. 2.** Extra-cellular material (arrows) surrounding a germ-tube (gt) with a side-branch and an appressorium (a). Bar = 1 μm. **Fig. 3.** A conidium of *A. cassiae* producing several germ-tubes at random points on the conidium body and at the tip of the filiform beak (arrow). Bar = 10 μm. **Fig. 4.** Branched (b) and unbranched germ-tubes of *A. cassiae* terminating in bulbous appressoria (a) or occasionally passing a stoma without appressorial formation (arrow). Bar = 10 μm. **Fig. 5.** A germ-tube of *A. cassiae* producing an intercalary appressorium (arrow). Bar = 10 μm. **Fig. 6.** An appressorium (a) forming on a stoma (s). Bar = 10 μm.

Figs 7-12. Scanning electron micrographs of the penetration events of *A. cassiae* on cowpea leaves. **Fig. 7.** A germ-tube (gt) entering a stoma (s), without appressorium formation. Bar = 10 μm. **Fig. 8.** The epidermis has been stripped off, showing the infection hyphae (h) penetrating through a stoma (s). Bar = 10 μm. **Fig. 9.** Bulbous primary hyphae (h) developing below the stoma (s) within the stomatal cavity. Bar = 10 μm. **Fig. 10.** The inner surface of the leaf, showing direct penetration of hyphae (h) through the epidermis after 72 hpi. Bar = 10 μm. **Fig. 11.** Hyphae (h) are visible within the mesophyll cells at 96 hpi. Bar = 10 μm. **Fig. 12.** Hyphae (h) growing intercellularly, passing through adjacent epidermal cells. Bar = 10 μm.

Figs 13-16. Light and scanning micrographs of the colonization events and structures of *A. cassiae* on cowpea. **Fig. 13.** A light micrograph of fungal structures of *A. cassiae* on the leaf surface. Central darkened area represents cells, which have discoloured as a result of the fungus. Bar = 10 μm. **Fig. 14.** By 168 hpi hyphae (h) of *A. cassiae* grew extensively and filled the epidermal cells of cowpea. Bar = 10 μm. **Fig. 15.** A young conidiophore (cp) of *A. cassiae* developing through a stoma of cowpea after 96 hpi. Bar = 10 μm. **Fig. 16.** Young conidiophores (cp) of *A. cassiae* emerging directly through the epidermis of cowpea at 96 hpi. Bar = 10 μm.
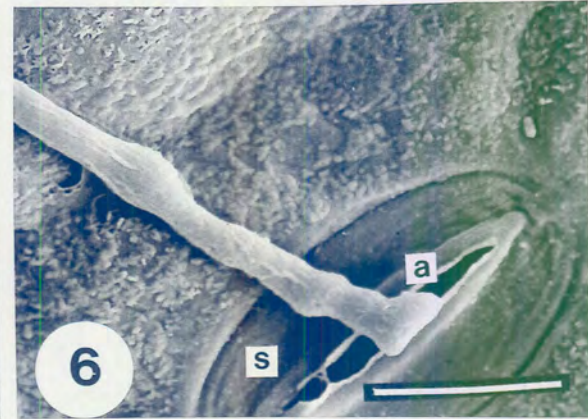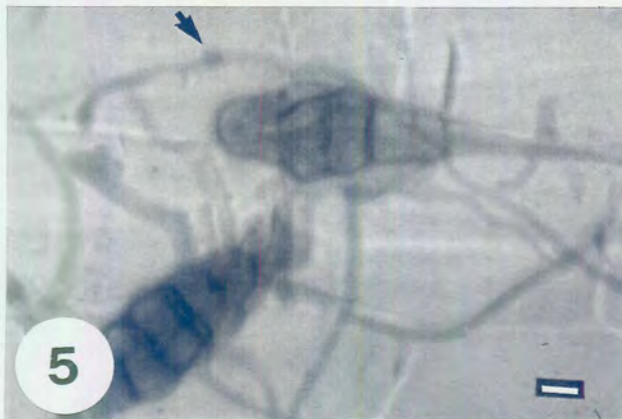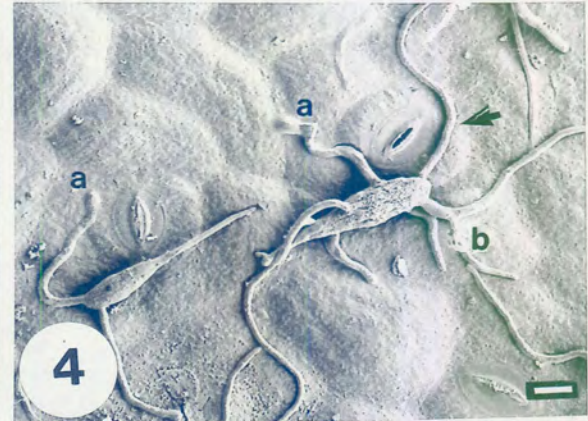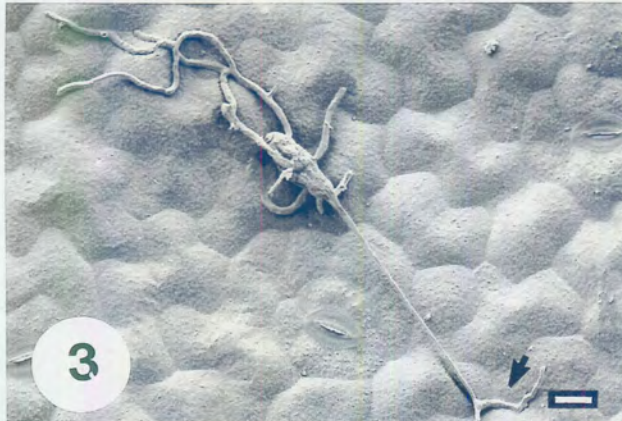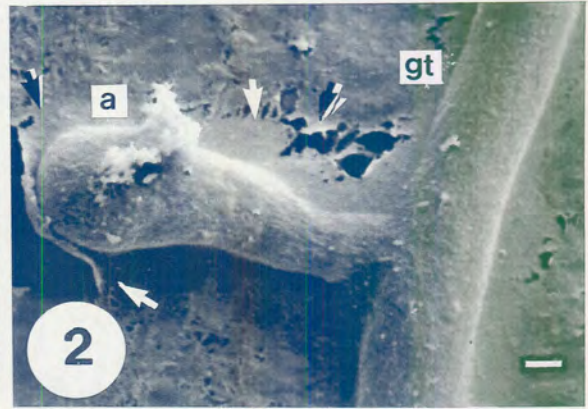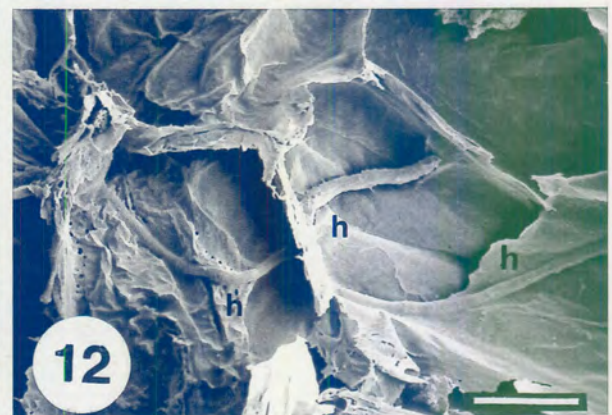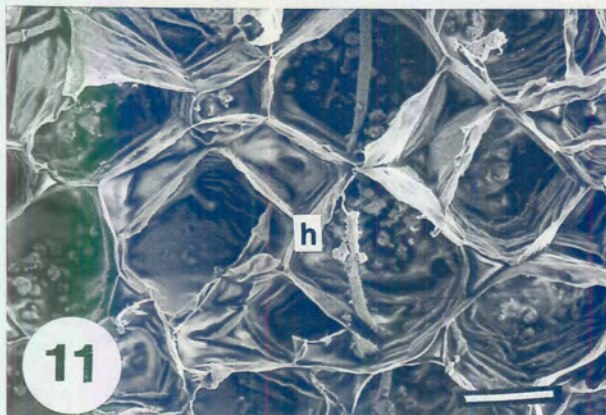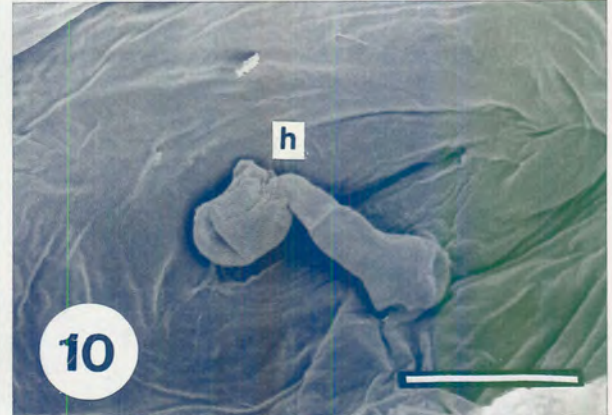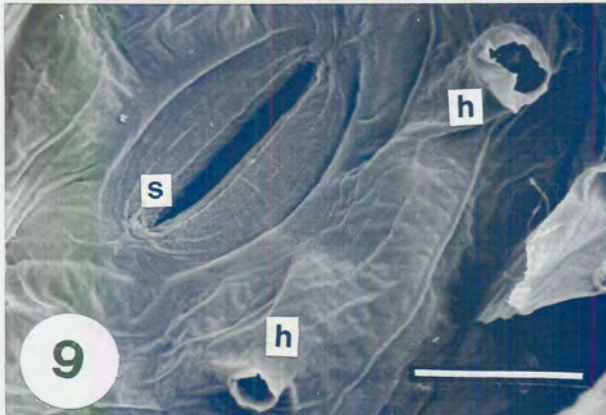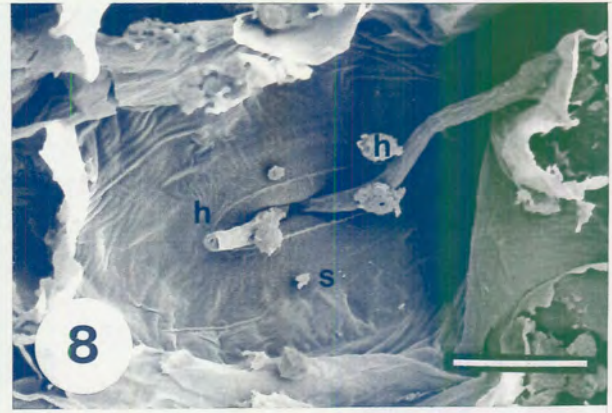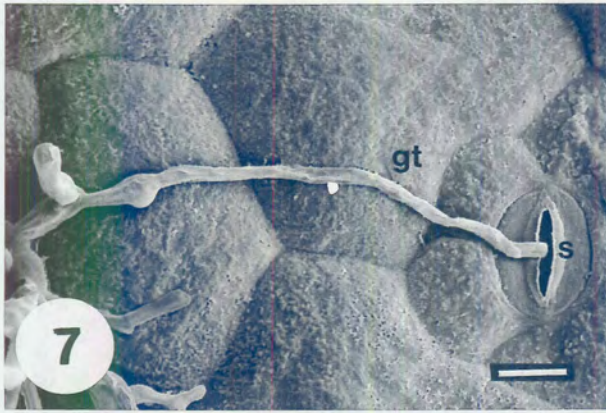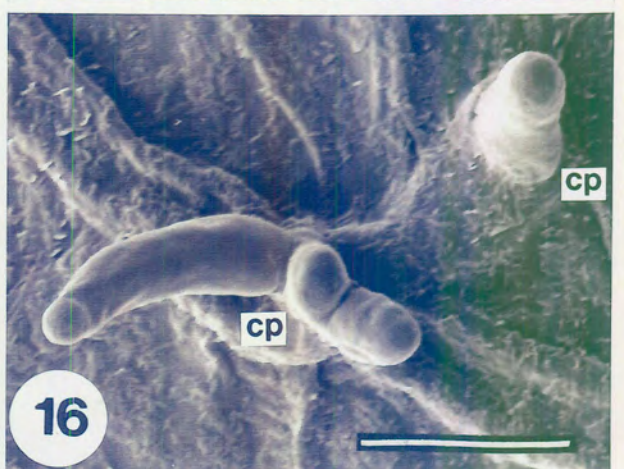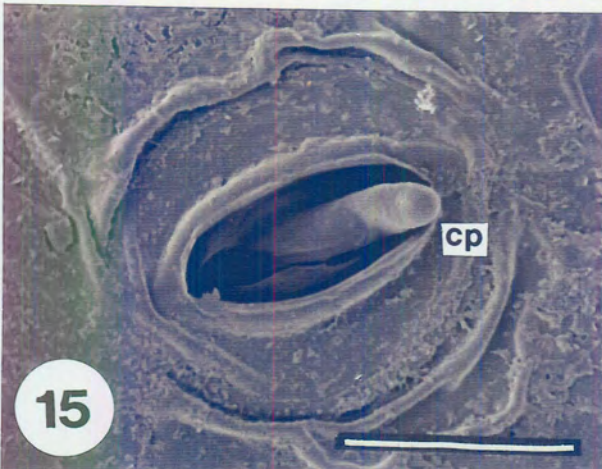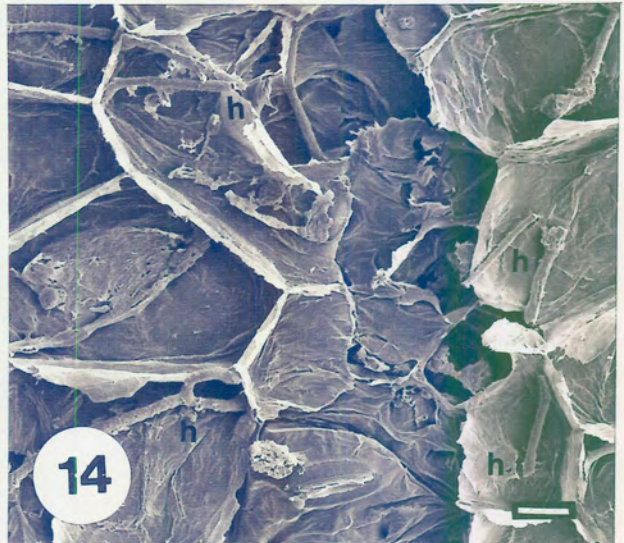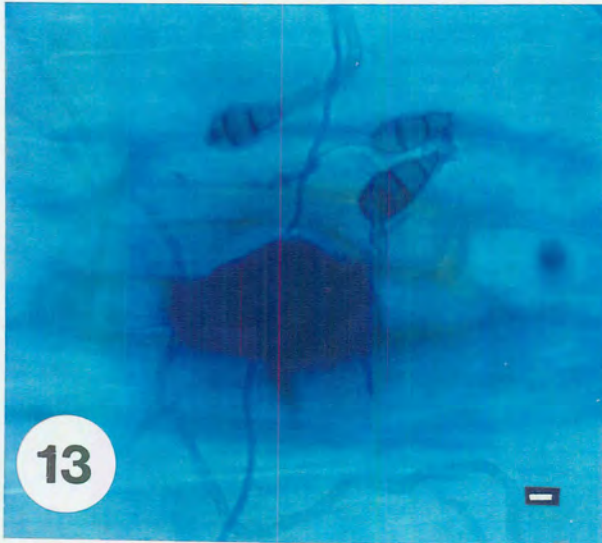
CHAPTER 6

CONIDIAL MORPHOLOGY AND DEVELOPMENT OF *ALTERNARIA CASSIAE*

Conidial morphology and development of *Alternaria cassiae*

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Key words: *conidial types, cowpea, culture media*

Abstract

Alternaria cassiae produced a mixed population of three distinct conidial types both *in vitro* and *in vivo*. Conidia with aseptate, long, filiform beaks were produced more frequently on all the media except PDA. Conidial body and beak sizes were variable when measured in culture and on cowpea leaves. Conidia produced *in vivo* were smaller in size, than those produced in culture. Conidia of *A. cassiae* were large, obclavate and formed singly or in chains. Conidiophores emerged directly through the epidermis or stomata. Hyphae growing on or above the leaf surface also differentiated into conidiophores. Conidiophores were straight or curved and enlarged apically at the site of conidium production. Smooth, bud-like conidial initials were produced singly at the apex of the conidiophores. As conidia matured, they became elliptical to obvate and densely verrucose. Once the mature conidium seceded, a small pore was visible at the apex of the conidiogenous cell.

Introduction

Conidia of *Alternaria cassiae* Juriar and Khan collected from cowpea plants (*Vigna unguiculata* (L.) Walp) in the field showed different conidial types when studied under the light microscope. In preliminary studies, we further noted that the conidial body and beak sizes of conidia produced *in vitro* and *in vivo* differed. Juriar and Khan (1960) gave the first species description of *A. cassiae* isolated from *Cassia holosericea* Fresen. In separate studies, Simmons (1982) and David (1991) described the morphological characteristics of the conidiophores and conidia of *A. cassiae* and further reported that *A. cassiae* produced a mixed conidium population, with three different conidial types on sicklepod (*Cassia* spp). These conidial types seem to have some diagnostic value, since few other *Alternaria* spp. display this feature. However, the formation of different conidial types may cause some confusion in the identification of a pathogen when cultures maintained on different media produce more, less or none of a specific conidial type. According to Rotem (1994), the dimensions of the spore body, including the beak, are considered some of the most important characteristics of a given species. Neergaard (1945) and Simmons (1967) also emphasised the role of conidial morphology for the description and identification of *Alternaria* spp. This study was done to determine the effect of different media on the conidial types and morphology of *A. cassiae* (isolated from cowpea) and to further describe the development of conidia of *A. cassiae* on the surface of diseased cowpea leaves using light microscopy and scanning electron microscopy (SEM).

Materials and methods

Fungal cultures

An isolate of *A. cassiae* (PPRI 6393) was obtained from the National Collection of Fungi, Pretoria, South Africa. The fungus was maintained on V8-agar and incubated at 25°C in a 12 h UV-light/12 h dark cycle. The following culture media were prepared aseptically to study the conidial types and morphology of *A. cassiae*: Potato dextrose agar (PDA) (Merck), malt extract agar (MEA) (Merck); water agar (WA) (Merck); cornmeal agar (CMA) (Merck); Czapek dox (CDA) (Oxoid); and V8-agar (V8-Juice; Campbell and agar; Merck). A plug of medium (7 mm in diameter) containing actively growing mycelium was removed aseptically from the periphery of a culture and placed inverted in the centre on each of six different culture media in 9 cm diameter plastic Petri dishes. Five replicates of each medium were used in the experiment and the plates were incubated in a 12 h UV-light/12 h dark cycle at 25°C for 18 days.

Plant material

Cowpea seeds (cv. Rhino) (two per pot) were planted in plastic pots (15 cm in diam.) containing pasteurised soil and maintained in a greenhouse at 25°C (±1°C). Plants were watered daily. The leaves of 6-8 week old seedlings were used for inoculation.

Inoculation procedure

Inoculum was prepared aseptically by pouring 2 ml sterile distilled water into Petri dishes containing a 14 d old sporulating culture and agitating it with a glass rod. Cowpea leaves were inoculated by painting them to run-off with a 1×10^5 conidia ml^{-1}

suspension of *A. cassiae* spores. Inoculated plants were placed in a mist chamber and kept at 25°C (±1ºC)until sampling.

Light microscopy

To study conidia types, morphology and development, leaves were cut into 5 mm^2 pieces, respectively at various time intervals after inoculation. The epidermis of specimens were stripped, mounted on microscope slides and stained with lactophenol blue. Micrographs were made with a Nikon Optiphot and a Zeiss photomicroscope. Mycelium was scraped from the six different sporulating media, stained with lactophenol blue, viewed with a Zeiss light microscope and photographed. The epidermis of cowpea leaves was stripped, mounted on slides and stained with lactophenol blue. Conidial types were described and conidium size was measured in μm, with the minimum, maximum and average size range being recorded. The conidial differentiation on the six different media and *in vivo* on cowpea leaves was expressed as a percentage.

Scanning electron microscopy (SEM)

To study conidial formation and detachment behaviour, 5mm^2 leaf pieces were cut from the inoculated plants at various time intervals after inoculation. Plant material was fixed in 2.5% (v/v) gluteraldehyde in 0.075 M phosphate buffer (pH 7.4-7.6). Material was rinsed in the same buffer and then post-fixed in 0.25% (w/v) aqueous osmium tetroxide for 2-4 h. This process was followed by three successive washing steps in distilled water. Material was dehydrated in an ascending ethanol series, critical point dried in a Bio-rad critical point dryer and mounted on stubs. All

specimens were coated with gold in a Polaron sputter coater and examined with a Jeol JSM 840 scanning electron microscope at 5 kV.

Results

Conidia of *A. cassiae* were large, obclavate and each conidium was divided by transverse and longitudinal septa into multiple compartments (Fig. 1). *A. cassiae* produced a mixed population of conidia both *in vitro* on all the different culture media and *in vivo* on the surface of diseased cowpea leaves. Conidia were borne singly (Fig. 10 & 11) or in chains of 2-4 conidia (Fig. 1). Three distinct conidial types were produced: 1) conidia with long filiform beaks (Fig. 2); 2) others that have generated secondary conidiophores with consequent chains of conidia (Fig. 1); and 3) those at full size and complete septation, but without beaks (Fig.10). Cultures grown on all the media except PDA, produced more conidia with long filiform beaks than those with short, or no beaks (Table 1). Conidia formed on CMA had the highest percentage of long beaked conidia, while conidia on PDA had the highest percentage of short beaked conidia (Table 1). Conidial body and beak sizes were variable in cultures grown on different media and on cowpea leaves (Table 1). All cultures grown on artificial media produced longer conidia than when formed on cowpea leaves *in vivo* (Table 1). Conidia formed on CDA had the largest conidum bodies (93x22 μm), and the shortest beak lengths, except for conidia on MEA (Table 1). Beaks of conidia produced on WA were the longest, reaching lengths of up to 330 μm (Fig. 2), whilst *in vivo* on cowpea leaves they reached lengths of up to 238 μm. The long filiform beaks were found to be aseptate (Fig. 2), while the short beaks that were converted into secondary conidiophores showed septation. Occasionally the tip of a filiform beak was converted into a slightly enlarged functional secondary

conidiophore, consequently giving rise to a chain of conidia (Fig. 1). Branched (Fig. 3) and unbranched (Fig. 4) septate conidiophores were produced in cultures on all the different culture media.

Conidial formation and detachment was studied *in vivo*. Seventy-two hours post inoculation (hpi), profuse fungal growth was observed on the leaf surface, but no conidiophores emerging through the epidermis were present. Conidiophores were formed in two ways in and around the lesions on the leaf surface. Firstly, hyphae growing on the plant surface differentiated into conidiophores (Fig. 5) by 24 hpi. Secondly, conidiophores emerged directly through the epidermis (Fig. 7 & 8) or through stomata (Fig. 6) 96 h after inoculation. By 168 hpi conidiophores were abundant and young developing conidia were present. Conidiophores formed on cowpea leaves were straight or curved and borne singly (Fig. 7) or in bundles (Fig. 8). The conidiophores were septate, branched (Fig. 7) or unbranched (Fig. 8) and 30-120 x 4-6 μm in size. Conidiophores were cylindrical in shape, but enlarged apically at the site of conidium production (Fig. 8). In most cases a single conidium was produced at the apex of a conidiophore (Fig. 9). Initially the conidium was round, bud-like and smooth (Fig. 9). Mature conidia were elliptical to obvate and densely verrucose (Fig. 10 & 11), with sizes ranging from 50-85 x 12-25 μm *in vivo*, excluding the length of the beak. Once the mature conidium seceded, a small pore (±0.5 μm) representing the conidiogenous locus, was visible at the apex of the conidiophore (Fig. 11). Occasionally secondary conidiophores were observed at the distal region of the existing conidiophore, this however only occurred when the mature conidium had already been detached (Fig. 12).

Discussion

As reported for *A. cassiae* isolated from *Cassia* spp. (Simmons, 1982; David, 1991), *A. cassiae* isolated from cowpea also produced a mixed conidium population of three conidial types both *in vitro* and *in vivo*. Typical long beaked conidia and conidia with shorter beaks, converted into secondary conidiophores were more frequently observed than mature beakless conidia. However, on PDA short beaked and beakless conidia were more prevalent. This may occasionally result in the incorrect identification of the fungus as another *Alternaria* spp. It is therefore necessary to define conidial sizes and types on specific media, to avoid confusion. The occurrence of several conidial types within one population, may possibly be linked to a mode of survival and dissemination.

An average body size (on all the different media) of 75 x 18μm was calculated for *A. cassiae*. This being slightly smaller than the 90 x 20 μm and 80-85 x 20-25 μm reported for *A. cassiae* by Simmons (1982) and Mims et al. (1997), respectively, but falls into sizes of 65-90 x 20 μm reported by David (1991).

Conidial beak lengths of up to 330 μm were observed in culture, and the average beak length on all the media was ±105 μm. According to Simmons (1982) and David (1991), beak lengths of 100-125 μm are more usual. The longest beak length reported by both these authors was 200 μm. The results obtained in this study revealed that WA, V8-agar and CMA more frequently produced conidia with conidial beaks longer than 105 μm. This might indicate that these media stimulate the development of long, filiform beaks.

Conidial formation and detachment was similar to other *Alternaria* spp. The emergence of conidiophores of *A. cassiae* through the epidermis and stomata as well

as the differentiation of hyphae, growing on the leaf surface, into conidiophores, indicated the initiation of sporulation. The induction of conidiophore formation from vegetative hyphae has also been reported in *A. cichorii* Nattrass (Kumagai, 1983). As was found in this study for *A. cassiae*, Allen et al. (1983) also observed two ways of conidiophore development. *A. helianthi* (Hansf.) Tubaki and Nishihara on sunflower (*Helianthus annuus* L.) produced conidiophores that protruded through stomata as well as other sites on the leaf, but conidiophores were also produced from mycelium growing across the leaf surface (Allen et al., 1983). Conidiophores were pigmented darker and morphologically different from vegetative hyphae, the latter being thinner. The lengths of conidiophores of *A. cassiae* in this study usually varied from 30-100 μm, corresponding well with the results obtained by David (1991), who reported conidiophore lengths of 36-112 μm.

Conidiophores of *A. porri* (Ellis) Cif. isolated from onion (*Allium cepa* L.) varied from 12-46 μm (Aveling et al., 1991). The tip of a conidiophore of *A. cassiae* was slightly swollen, where the conidium protruded. These results were very similar to that of *A. porri* by Aveling et al. (1991), who furthermore found that the conidial initial of *A. porri* on onions was a bud-like protrusion. The conidial initial of *A. cassiae* was also bud-like and appeared smooth to slightly verrucose. David (1991) also described the young conidia as being smooth-walled and slightly verrucose. Our results, furthermore, indicated that the conidia became densely verrucose as they matured. The presence of transverse and longitudinal septa in the conidia of *A. cassiae* on cowpea leaves, were distinct characteristics of mature conidia. Aveling et al. (1991) also reported that transverse and often longitudinal septa were present in mature conidia of *A. porri*. Simmons (1982) reported that mature conidia of *A.*

conidium a surface view of 12-20 compartments.

Characteristic conidiophore proliferation, as described by Simmons (1982), was observed occasionally. This however, was only observed after a mature conidium was dislodged. In this study, as described for *A. porri* on onion (Aveling et al., 1991), a tiny pore, representing the conidiogenous locus was visible at the apex of the conidiophore, once the mature conidium had been detached.

In mycological literature, the morphological characters of fungi are often only described on one or two different media. Consequently, when identification of a fungus is attempted, it is often difficult to determine whether observed differences are due to innate differences in the fungus or to the effect of the media. This study therefore attempted to provide a more comprehensive morphological description of conidial types of *A. cassiae* on different media and their development, making use of light microscopy and scanning electron microscopy.

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Table 1.   Spore dimensions of *A. cassiae in vitro* and *in vivo*.

Measurements (μm)[a]	*In vivo*[b]	CDA	CMA	MEA	PDA	V8-juice	WA
Spore length:							
Mean	63	93	86	67	65	69	72
Range	50-85	80-110	62-103	53-87	53-81	50-85	62-87
S.D. [c]	8.56	8.24	7.92	7.92	7.01	6.37	6.07
Spore width:							
Mean	17	22	20	17	17	17	15
Range	13-25	19-25	12-25	15-22	12-22	12-23	12-25
S.D.	2.85	4.67	2.97	2.07	1.92	1.96	2.47
Beak length:							
Mean	128	74	111	66	90	108	179
Range	10-238	56-87	18-198	10-260	18-173	10-229	24-330
S.D.	56.64	15.19	31.35	62.48	35.16	49.08	72.16
Percent of spores with:							
No beaks	8	15	11	6	11	17	7
Short beaks	30	31	21	38	66	30	31
Long beaks	62	54	68	56	23	53	62

[a] Each figure (except CDA and MEA) is the average of measurements of 200 spores of *A. cassiae*. Each figure in the CDA and MEA columns is the average of measurements of 100 spores.

[b] Conidia measured *in vivo* on cowpea leaves

[c] Standard deviation

Legends to figures

Fig. 1. Large conidium (c) of *A. cassiae* with transverse and longitudinal septa and a long filiform beak, which is converted into a secondary conidiophore giving rise to a chain of one conidium (c). Bar = 10μm. Fig. 2. A conidium of *A. cassiae*, produced in culture on water agar, with a long aseptate, filiform beak. Bar = 10μm. Fig. 3. A branched conidiophore of *A. cassiae* produced in culture. Bar = 10μm. Fig. 4. An unbranched conidiophore of *A. cassiae* produced in culture. Bar = 10μm.

Fig.5. A hyphae (h) of *A. cassiae* growing on the leaf surface differentiated into a conidiophore (cp). Bar = 10μm. Fig. 6. A conidiophore (cp) of *A. cassiae* emerging through a stoma (s). Bar = 10μm. Fig. 7. A single branched conidiophore of *A. cassiae* (cp) emerging directly through the epidermis. Bar = 10μm. Fig. 8. Cylindrical, unbranched conidiophores (cp) of *A. cassiae* grouped in a bundle. An enlarged apex (arrow) is visible at the site of conidium production. Bar = 10μm.

Fig. 9. A young smooth, bud-like conidium (c) of *A. cassiae* developing at the apex of a conidiophore (cp). Bar = 1μm. Fig. 10. Mature and densely verrucose beakless conidium (c) of *A. cassiae* with a broader base than the apex. Bar = 10μm. Fig. 11. A mature, verrucose conidium (c) of *A. cassiae* is being detached and a pore is visible at the apex of the conidiophore (arrow). Bar = 10μm. Fig. 12. Formation of a secondary conidiophore (scp) of *A. cassiae* after the mature coniduim has been detached. Bar = 10μm.
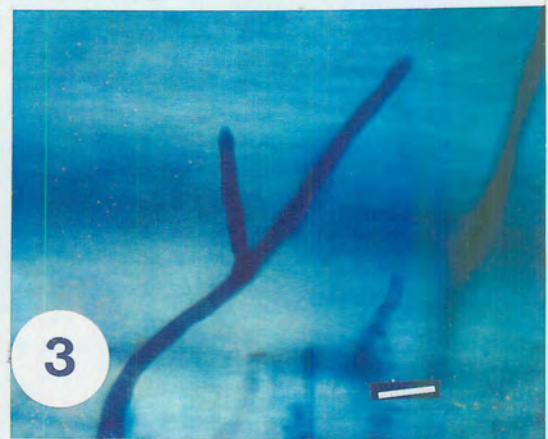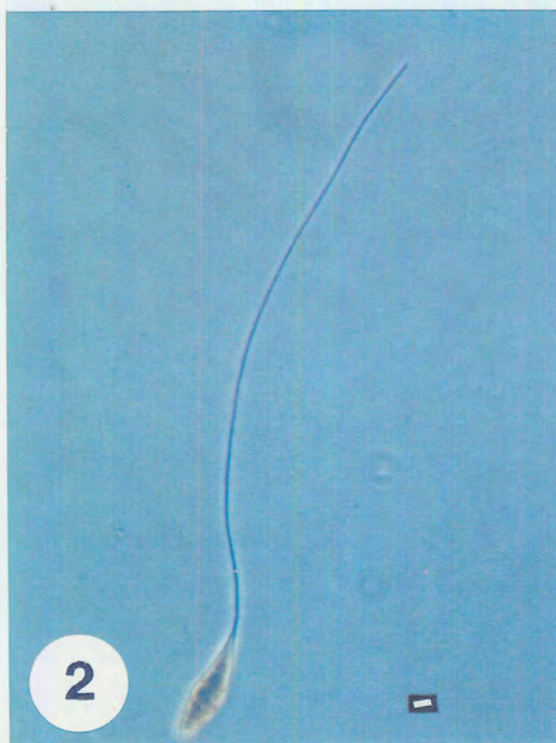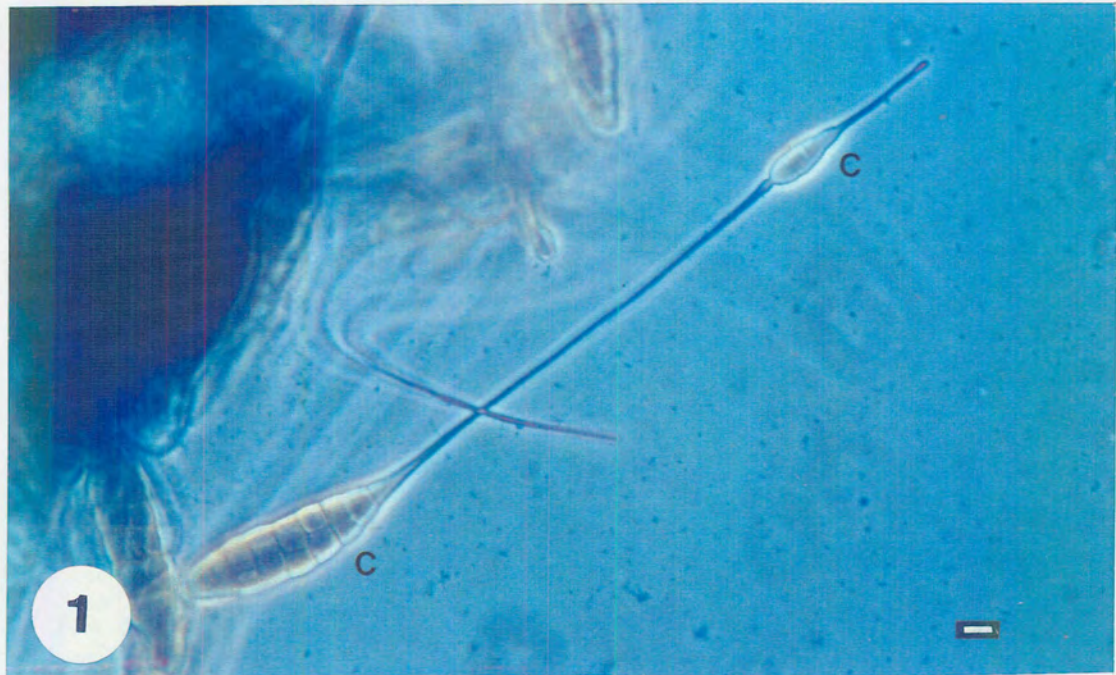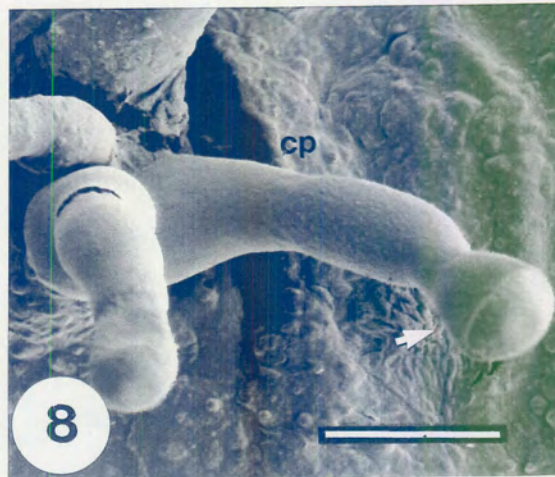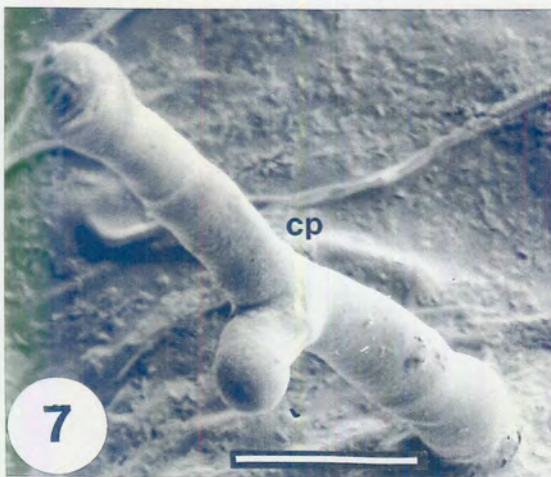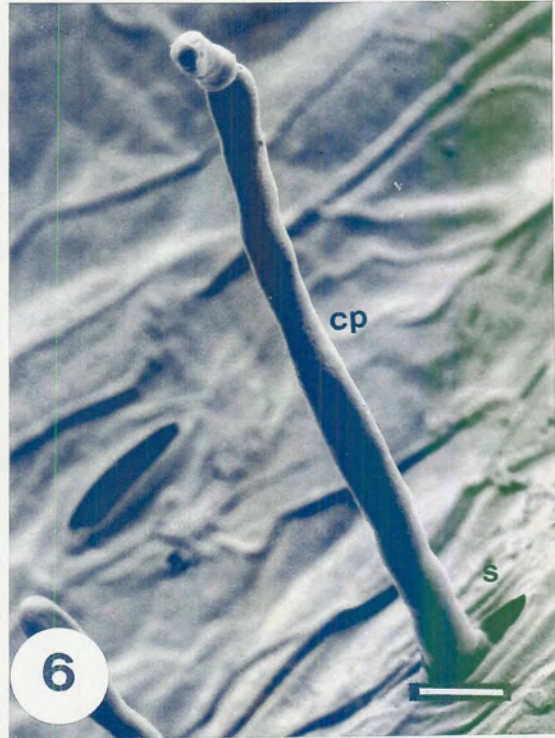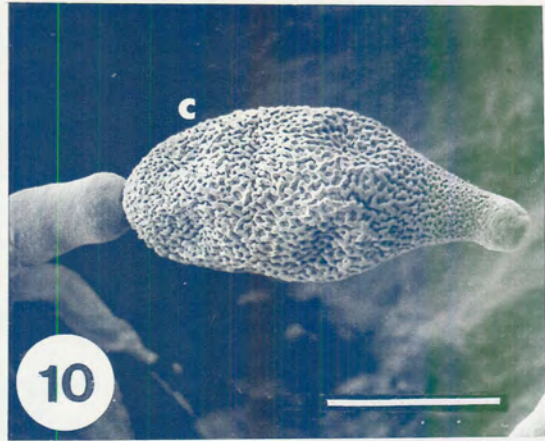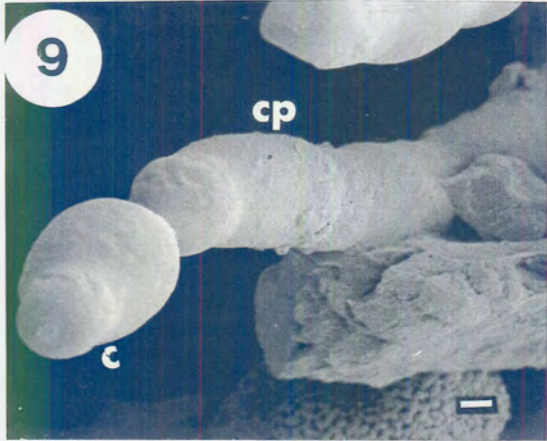
CHAPTER 7

THE EVALUATION OF SIX FUNGICIDES FOR REDUCING *ALTERNARIA CASSIAE* ON COWPEA SEED

The evaluation of six fungicides for reducing *Alternaria cassiae* on cowpea seed.

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ABSTRACT

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Alternaria cassiae Juriar & Khan was found to be a destructive seed-borne, foliar pathogen of cowpea (*Vigna unguiculata* (L.) Walp) in Southern Africa. Six fungicides at three concentrations were evaluated for their efficacy in reducing the pathogen in culture and on seed. The fungicides included benomyl, bitertanol, captab, mancozeb, propiconazole and triforine. An untreated control was included for comparison. Seeds were artificially inoculated with *A. cassiae*, treated with the different fungicides and percentage germination and infection was determined *in vivo*. Percentage emergence and disease incidence was also determined in greenhouse experiments. None of the treatments eradicated *A. cassiae* from cowpea seeds. Captab at 1.5x the recommended rate proved to be the best treatment for reducing the pathogen.

Cowpea (*Vigna unguiculata* (L.) Walp) is an important food and fodder crop and is increasingly being cultivated by small-scale farmers in South Africa (22). Cowpea seeds are particularly rich in protein and fulfil in many of the dietary needs of rural people (15). However, there are several reports of seed-borne fungi associated with cowpea diseases (4,14,19). Therefore, the need arises for research on diseases of cowpea, especially those transmitted via seed and those resulting in significant yield losses. Field surveys of cowpea growing areas in Southern Africa revealed the presence of a destructive foliar disease. *Alternaria cassiae* Juriar & Khan was reported as the causal pathogen of this new disease on cowpea (8). The pathogen has previously been recorded on *Cassia* spp., *Rhynchosia* sp. and *Crotolaria* sp. (3,7,23).

Alternaria spp., in general, are seed-borne (12). *A. porri* (Ellis) Ciferri is seed-borne in onion (11,17). Singh *et al.* (18) found that *A. tenuis* Nees is present in all layers of the pericarp and also invades the endosperm and embryo of sunflower seeds. Maude & Humpherson-Jones (10) reported that *A. brassicicola* (Schwein.) Wiltshire was present on brassica seed and *A. sesamicola* Kawamura on sesame seed. To date little research has been done in South Africa to evaluate seed treatments for the control of seed-borne diseases of cowpea. Smith *et al.* (21) evaluated eight chemical fungicides and one biocontrol method for the treatment of *Colletotrichum dematium* (Pers) Grove on cowpea. The objectives of this study were to determine whether *A. cassiae* is seed-borne in cowpea, to test various chemical fungicide treatments for control of *A. cassiae in vitro* and *in vivo*, and to evaluate the most promising treatments in the greenhouse.

MATERIALS AND METHODS

Standard agar seed health test. The standard agar seed health test according to the rules of the International Seed Testing Association (ISTA) (5) was used to determine whether *A. cassiae* is seed-borne in cowpea. Two cowpea cultivars (Rhino obtained from Agricultural Research Council –Grain Crop Institute and IT82D889 obtained from International Institute for Tropical Agriculture) were evaluated. Four replicates of 100 seeds of each cultivar were surface sterilized in 1% (w/v) sodium hypochlorite for two minutes. Seeds were plated out (10 per Petri dish) onto cornmeal agar (CMA) amended with 0.025% chloramphenicol and incubated at 25°C under a 12h UV-light/12h dark regime for 7 days. The seeds were examined for fungal growth using a Nikon SMZ 645 stereomicroscope. The species identification of *A. cassiae* was confirmed by staining conidia with lactophenol blue and viewed with a Zeiss Standard 20 light microscope. Conidia were compared with the isolate PPRI 6393 obtained from the National Collection of Fungi, Plant Protection Research Institute, Pretoria. The cultivar with the highest infection of *A. cassiae*, IT82D889, was used for subsequent experiments.

Fungicide treatments. Fungicides were used at the registered rates in the *in vitro* and *in vivo* experiments. They are listed by common name and active ingredient (the trade name, manufacturer and recommended dosage are given in parentheses). The fungicides were: benomyl WP, 500g/kg a.i. (Benlate, du Pont, Halfway House, RSA – 5g/10ℓ); captab 80% WG, 500g/ℓ a.i. (Captan, Kombat, Greytown, RSA – 20g/10ℓ); propiconazole, 500g/ℓ a.i. (Novel, Novartis, Isando, RSA – 2.5ml/10ℓ); mancozeb, 800g/kg a.i. (Dithane M45, Starke Ayres, RSA – 20g/10ℓ); bitertanol, 300g/ℓ a.i. (Baycor, Bayer, Isando, RSA – 4ml/10ℓ) and triforine, 190g/ℓ a.i. (Denarin,

Cyanamid, RSA – 15ml/10*l*). The above rates were also used as standard rates per litre of medium in the *in vitro* experiment.

In vitro **experiment.** An isolate of *A. cassiae* (PPRI 6393) was maintained on V8-agar at 25°C in a 12h UV-light/12h dark cycle. The six test fungicides, at 0.5x, 1x and 1.5x the registered rate, were each incorporated into CMA after autoclaving. Discs (7mm diameter) from actively growing cultures of the isolate were transferred aseptically to the centre of amended plates. Control plates containing no fungicides were included in the experiment. Four replicate plates of each treatment were incubated at 25°C in 12h UV-light/12h dark. Colony diameter was measured after 5 and 8 days. Data was analysed using the analysis of variance test, and significant differences were determined using the Student's T-test at $P = 0.05$.

In vivo **experiment.** Eleven samples of 100g of cowpea seed (IT82D889) were artificially inoculated with *A. cassiae* by slurry coating with a 10^5 conidia ml^{-1} water spore suspension and left to air dry in a laminar flow cabinet. Based on the results obtained from the *in vitro* experiment the ten best fungicide treatments at the tested concentrations were used in this experiment. All three test concentrations of mancozeb and propiconazole, 1.0x and 1.5x triforine, 1.5x bitertanol and 1.5x captab were used. Each of the chemicals was suspended in 5ml sterile distilled water and applied as a slurry to the seed samples. The control was also inoculated with *A. cassiae* as previously described and treated with 5ml sterile distilled water.

Seed germination assays. The effect of the different treatments on percentage germination was determined. Three replicates of 50 treated seeds from each chemical treatment were placed on moist Whatman No.1 blotter paper in Petri dishes, sealed with parafilm, and incubated at 25ºC in a 12h UV-light/12h dark regime. Percentage germination was rated on day five and eight. Data was analysed as previously described.

Agar seed health test. The standard agar seed-health test was conducted (5). Four replicates of 100 seeds from each of the previously described treatments were used. Seeds were plated onto CMA supplemented with 0.025% chloramphenicol (10 seeds/plate). Plates were incubated at 25ºC in a 12h UV-light/12h dark regime. Seeds were observed under a dissecting microscope for the presence of fungal growth.

Greenhouse trial. Based on the results of the *in vivo* seed germination and agar seed health tests, three replicates of 20 seeds of the eight best treatments used in the *in vivo* experiments were planted in seedling trays containing a peat-based growing medium. Plants were maintained in a glasshouse (18/25°C (±1ºC) night/day temperature) and watered daily. The treatments were; bitertanol 1.5x, captab 1.5x, mancozeb 1.0x and 1.5x, propiconazole 1.0x and 1.5x and triforine 1.0x and 1.5x. The control sample was treated with sterile distilled water. Seven weeks after planting, percentage emergence, shoot and root length and percentage abnormality (abnormalities such as chlorosis, deformed roots, stems or leaves and stunted growth) and disease incidence of seedlings was determined. Experimental layout in the greenhouse was a randomised block design repeated once. Data was analysed as previously described.

RESULTS

Standard agar seed health test. *Alternaria cassiae* was found to be seed-borne on Rhino and IT82D889 cowpea seeds, with respective infection percentages of 6 and 14%. As IT82D889 cultivar seeds had the highest infection of *A. cassiae,* this cultivar was used for further experiments.

In vitro **experiment.** All fungicides except Benomyl at 0.5x, 1x and 1.5x significantly inhibited mycelial growth of *A. cassiae* in culture (Table 1). All the plates amended with benomyl showed a significantly higher rate of fungal growth than the control plates (Table 1). It is evident from Table 1 that mancozeb and propiconazole at all concentrations tested, totally inhibited mycelial growth. Triforine at 1x and 1.5x the registered rate was also effective in inhibiting the growth of the fungus, a very slight growth of 0.5mm and 0.25mm, respectively, was measured (Table 1).

In vivo experiments.

Seed germination assays. Only bitertanol at 1.5x the recommended dosage significantly reduced percentage germination of cowpea seed when compared to the control (Table 2).

Agar seed health test. All the fungicides tested significantly decreased the percentage of *A. cassiae* infection of cowpea seeds artificially inoculated with the pathogen (Table 2). Bitertanol 1.5x, captab 1.5x, mancozeb 1.5x, propiconazole 1.5x and triforine 1.5x were most effective in reducing the incidence of *A. cassiae* (Table 2).

Greenhouse trial. All treatments except bitertanol 1.5x, captab 1.5x and mancozeb 1.0x, significantly decreased the percentage emergence when compared to the control (Table 3). None of the treatments except bitertanol 1.5x showed a difference in shoot and root length when compared to the control (Table 3). Propiconazole 1.5x showed a significant decrease in root length when compared to the control (Table 3). Bitertanol 1.5x, mancozeb 1.0x and triforine 1.5x showed a significantly higher percentage abnormality. Percentage disease incidence was significantly reduced by all treatments, except triforine 1.0x and 1.5x, when compared to the control (Table 3).

DISCUSSION

A. cassiae was found to be seed-borne in cowpea seeds. Thirty-six seed borne *Alternaria* spp. have been listed (12) and according to Rotem (16), this might suggest that practically all *Alternaria* spp. pathogenic to foliage, also infect seeds.

According to Neergaard (12), effective seed treatments must eliminate pathogens without being toxic to seeds. All the fungicides tested in the *in vitro* study, except the three concentrations of benomyl were effective in significantly inhibiting mycelial growth of *A. cassiae* in culture. According to Smith *et al.* (21) benomyl was not very effective in inhibiting growth of *Colletotrichum dematium* also isolated from cowpea when tested *in vitro.* However, Aveling *et al.* (2) reported that benomyl at 1.0x and 1.5x the recommended rate significantly inhibited the mycelial growth of *Alternaria porri.* According to Sivapalan (20), benomyl significantly reduced the germination of conidia of *Alternaria brassicicola in vitro,* when compared to the control. Benomyl is a systemic benzimidazole based fungicide effective against a

large number of fungal pathogens (9), however this group of fungicides has no effect on some of the dark-spored imperfect fungi, such as *Helminthosporium* spp., *Phoma* spp. and *Alternaria* spp. (1,9). Mancozeb and propiconazole at all the tested concentrations were most effective in preventing fungal growth of *A. cassiae in vitro*. Sivapalan (20) reported a significant reduction in the germination of conidia of *A. brassicicola* treated *in vitro* with mancozeb.

Bitertanol, captab, mancozeb, propiconazole and triforine at 1.5x the recommended rates were all effective in reducing the percentage infection of seeds artificially inoculated with *A. cassiae*. Captab and mancozeb are protective, non-systemic fungicides and thus prevent fungal growth on the external surface of seeds therefore inhibiting further infection of the seed (21). Bitertanol and propiconazole are systemic fungicides and are often applied as seed or soil treatments. According to Agrios (1) both bitertanol and propiconazole show long protective and curative activity against the imperfect fungi, such as *Alternaria* spp. Triforine is a systemic fungicide and is effective against many ascomycetous and imperfect fungi. In South Africa mancozeb, captab (captan) and triforine are some of the fungicides generally recommended for the control of alternaria diseases on vegetables, fruits and flowers (13).

None of the fungicides eradicated *A. cassiae* from cowpea seed in the *in vitro* experiment indicating that they are possibly not fungitoxic to the pathogens or did not penetrate the seed tissue to kill internal mycelium.

In the greenhouse, all treatments, except triforine 1.0x and 1.5x significantly decreased the disease incidence of cowpea seedlings when compared to the control. Seeds treated with mancozeb showed the lowest disease incidence. However a significant increase in abnormality and a decrease in emergence was also recorded.

Sivapalan (20) reported that broccoli seed artificially inoculated with *A. brassicicola* showed a higher percentage emergence and a lower percentage fungal infection when treated with mancozeb.

Of all the fungicides tested, captab at 1.5x the recommended rate was the best treatment overall. According to Wu, Wu & Wu (24) captan at 500ppm was able to completely inhibit the germination and sporulation of *A. brassicicola*. Captab also showed promising results when tested for the control of *C. dematium* on cowpea (21). Captab has been commercially used as a seed and soil treatment since the 1950's (6) and has remained on the market since then. In South Africa, captab (captan) is registered on many vegetables and fruits, but it is, as yet, not registered on cowpea. This fungicide looks promising as a seed treatment for cowpea, but will need to be tested under field conditions.

Currently, there are no fungicides registered as seed treatments for cowpea in South Africa. However, a major role of seed treatments is the disinfection of seeds, thus preventing the local and international spread of infected seed samples. The results presented in this study provide valuable data, which may assist in the registration and development of a seed treatment for cowpea.

ACKNOWLEDGEMENTS

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Table 1. Colony diameter of *Alternaria cassiae* grown on cornmeal agar amended with various fungicides.

Treatment	Diameter of colonies (mm)
Control	28.25 *g
Benomyl 0.5x	34.0 i
Benomyl 1x	31.25 h
Benomyl 1.5x	31.5 h
Bitertanol 0.5x	7.63 ef
Bitertanol 1x	4.5 c
Bitertanol 1.5x	2.5 b
Captab 0.5x	8.5 f
Captab 1x	6.25 de
Captab 1.5x	2.5 b
Mancozeb 0.5x	0 a
Mancozeb 1x	0 a
Mancozeb 1.5x	0 a
Propiconazole 0.5x	0 a
Propiconazole 1x	0 a
Propiconazole 1.5x	0 a
Triforine 0.5x	5.13 cd
Triforine 1x	0.5 a
Triforine 1.5x	0.25 a
LSD	1.585

*Each value is the mean of four plates measured after eight days' growth. Values within a column not followed by the same letter are significantly different ($P = 0.05$) according to the Student T-test.

Table 2.   Percentage germination and infection of cowpea seed by *Alternaria cassiae* treated with various fungicides.

Treatment	Germination (%)	*A. cassiae* infection (%)
Control	66 *b	42 e
Bitertanol 1.5x	47 a	5 a
Captab 1.5x	62 b	7 ab
Mancozeb 0.5x	64 b	19 cd
Mancozeb 1.0x	57 ab	14 abc
Mancozeb 1.5x	72 b	7 ab
Propiconazole 0.5x	62 b	24 d
Propiconazole 1.0x	67 b	15 bcd
Propiconazole 1.5x	69 b	8 ab
Triforine 1.0x	61 ab	13 abc
Triforine 1.5x	61 ab	5 a
LSD	14.15	9.979

*Each value is the mean of 4 replications of 100 seeds. Values within a column not followed by the same letter are significantly different ($P = 0.05$) according to the Student T-test.

Table 3. Effect of six fungicide treatments of cowpea seed on percentage emergence, shoot and root length, percentage abnormality and diseased seedlings.

Treatment	Emergence (%)	Shoot length (mm)	Root length (mm)	Abnormality (%)	Disease (%)
Control	75.00 *c	305.3 bc	106.3 c	10.33 a	33.67 b
Bitertanol 1.5x	64.67 bc	253.3 a	88.3 ab	30.00 c	16.67 a
Captab 1.5x	69.00bc	310.0c	106.0 c	15.00 ab	18.00 a
Mancozeb 1.0x	69.00bc	275.7 abc	98.67 bc	24.33 bc	18.00 a
Mancozeb 1.5x	54.33 a	274.3 abc	98.33 abc	15.00 ab	12.50 a
Propiconazole 1.0x	50.00 a	280.7 abc	93.33 abc	11.5 ab	15.67 a
Propiconazole 1.5x	58.67 ab	264.3 ab	82.33 a	21.00 abc	15.00 a
Triforine 1.0x	56.33 ab	278.3 abc	92.33 abc	21.00 abc	21.67 ab
Triforine 1.5x	50.00 a	279.7 abc	93.33 abc	24.00 bc	21.33 ab
F-value	4.358	1.471	2.209	2.733	2.231

*Each value is the mean of 3 replications of 20 seeds. Values within a column not followed by the same letter are significantly different ($P = 0.05$) according to the Student T-test.

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CHAPTER 8

GENERAL DISCUSSION

Cowpea is an important, indigenous crop in Africa, and it is of major importance to the livelihoods of millions of people in rural areas (Quin 1997). The crop has shown potential for successful cultivation in areas of low agricultural potential. The Resource Poor Agriculture programme of the Agricultural Research Council, Roodeplaat - Vegetable and Ornamental Plant Institute, is currently running an "Indigenous Vegetable Crops" project aimed at studying the agronomic aspects of these crops in order to optimise production under local conditions in South Africa. A new discipline has recently been introduced to the project, focussing on the diseases affecting indigenous crops. This resulted in a collaborative cowpea disease program with the University of Pretoria, South Africa. The program aims at providing information on the epidemiology, symptomatology and control of cowpea diseases in South Africa.

In South Africa, cowpeas are being grown on a larger scale in the Northern Province, Mpumalanga, North West Province and KwaZulu-Natal (Coetzee 1995; Van den Heever *et al.* 1996). Small-scale and emerging farmers, in particular, are growing cowpeas more intensively (Coetzee 1995). The cultivation of any crop in great abundance or in monoculture increases the potential threat of pests and diseases. The need therefore arises to study these diseases that might affect the cowpea crop.

A survey of cowpea growing areas revealed the presence of a destructive foliar disease, particularly in the warm areas where excess rain was recorded during the growing season. This disease caused by *Alternaria cassiae* Juriar & Khan, is a first report on cowpea in South Africa and the world (La Grange & Aveling 1998). *A. cassiae* was first isolated from *Cassia holosericea* Fresen (Juriar & Khan 1960) and is

mainly associated with *Cassia* spp. (David 1991). The recording of a new disease provides many challenging research opportunities. This study aimed at providing new information on the disease and the causal pathogen, *A. cassiae*. The objectives of this study were to describe the symptoms of the disease (Chapter 3), evaluate the effect of culture media and other factors on the growth and sporulation of *A. cassiae* (Chapter 4), determine the penetration and infection process (Chapter 5) and to study the morphology and conidial development of *A. cassiae* (Chapter 6). *A. cassiae* was found to be seed-borne and various fungicides were evaluated for their efficacy in controlling the pathogen on cowpea seed (Chapter 7).

A. cassiae is prevalent under warm conditions, and the disease is particularly severe if cowpea is grown under monoculture and spreads rapidly under wet conditions. Symptoms on the leaves are characterised by black velvet masses of conidia that are visible on the necrotic tissue.

Each fungus has specific *in vitro* requirements for optimal growth and sporulation. Fungal growth and sporulation of *A. cassiae* was significantly higher on V8-agar. The minimum temperature for fungal mycelial growth was 10°C and the lethal maximum temperature was higher than 40°C. Optimal growth was obtained between 20 and 25°C. A 12h UV-light/12h dark cycle significantly enhanced sporulation of *A. cassiae*, while the 24h UV-light cycle inhibited the production of conidia. Wounding of the cultures further enhanced sporulation of *A. cassiae* maintained in culture.

Alternaria spp. generally exhibit similar infection strategies (Rotem 1994). Until now, only one study has been conducted on the infection process of *A. cassiae* on

sicklepod. No studies have been done on the infection and penetration of any *Alternaria* spp. on cowpea. Knowledge of infection processes and structures is required for a better understanding of the pathogen and the host reaction to infection. This could assist the researcher to develop appropriate forecasting models, control strategies and agronomic practices.

Conidia of *A. cassiae* germinated within 2-3 hpi, producing several germ-tubes, as is the case with many *Alternaria* spp. (Rotem 1994). Large amounts of extra-cellular material was observed in the vicinity of conidia and their germ-tubes. This could possibly play an important role in the adherence of the pathogen to the host. Branched, and unbranched germ-tubes usually terminated in appressoria, forming on epidermal cells or over stomata. Intercalary appressoria were also occasionally formed. Penetration through stomata, without appressorial formation also occurred. *A. cassiae* enters the host tissue both directly and indirectly, indicating that the pathogen is not necessarily reliant on wounds or natural openings, such as stomata for penetration. Necrotic host cells were present, even prior to penetration, as reported for *A. cassiae* on sicklepod (Van Dyke & Trigiano 1987). After penetration bulbous primary hyphae were produced within the epidermal cells. Secondary hyphae grew within intercellular spaces, penetrated and grew within epidermal and mesophyll cells. *A. cassiae* grew profusely and colonised the host tissue by 168hpi. Conidiophores emerged through the epidermis and stomata of necrotic leaves. By 288hpi black velvet masses of conidia were present on the leaf surface. From the results it is concluded that *A. cassiae* is a virulent, necrotrophic pathogen.

A. cassiae (isolated from cowpea) produced three distinct conidial types in culture on different media and on cowpea leaves. These mixed populations of conidia have also previously been reported for *A. cassiae* isolated from sicklepod (*Cassia* spp.) (Simmons 1982; David 1991). Conidia with long filiform beaks, reached lengths of up to 330μm when maintained on WA. Conidia with shorter beaks, converted into secondary conidiophores were also frequently observed, while mature beakless conidia were only occasionally seen. Occasionally the tip of a long filiform beak was slightly enlarged, forming a secondary conidiophore, consequently giving rise to a chain of conidia. Conidial chains were made up of 2-4 conidia.

Conidia of *A. cassiae* produced in culture were larger than those produced on cowpea leaves. In culture sizes ranged from 50-110x12-25μm, while the average sizes of conidia produced *in vivo* was 63x17μm. Conidia were large, obclavate and divided into multiple compartments.

When studying conidium formation in fungi, the use of transmission electron microscopy techniques is necessary for a complete understanding of the process. However, even though this study only made use of light microscopy and scanning electron microscopy it nevertheless provided useful information on the conidial development of *A. cassiae*, which could prove to be valuable in future studies. Conidiophores developed in one of two ways, firstly mycelia growing on the leaf surface differentiated into conidiophores or secondly, conidiophores emerged directly through the epidermis or stomata. Conidiophores enlarged apically at the site of conidium production, producing a bud-like conidial initial. As conidia mature, they become elliptical to obvate and densely verrucose. Once the mature conidium had

been detached, a small pore was visible at the apex of the conidiophore. Occasionally secondary conidiophores were formed at the distal region of the first conidiophore.

A. cassiae was found to be seed-borne in cowpea. The association of a pathogen with the seed of any crop causes problems with crop management and results in wide dissemination of the disease. Reducing the occurrence of or eradicating *A. cassiae* in or on cowpea seed would curtail the introduction of the disease into the cowpea crop and into new cowpea growing areas. Consequently, disease expression in mature plants should be less severe and grain yield and quality less reduced. Control of cowpea diseases can be achieved by biological, chemical, cultural, genetic and integrated management strategies. However, in South Africa, we must bear in mind that the majority of cowpea growers are small-scale and emerging farmers who cannot afford expensive control methods. The development of cowpea varieties resistant to *A. cassiae* would be a low cost solution for the farmer, it would however be a expensive and time consuming process, but would provide a potentially long-term solution. Cheaper control methods such as crop rotation, intercropping, the use of healthy and/or treated seed and basic field sanitation are probably the most effective methods to prevent and control this disease.

Of the six fungicides evaluated as possible seed treatments, the active ingredient captab, over all, proved to be the most effective in reducing *A. cassiae in vitro* and on artificially inoculated seed. Captab is as yet not registered on cowpea in South Africa, but it is registered on other vegetable crops for disease control. Captab has potential as a seed treatment for cowpea and possibly other legumes too. However, the usefulness of any chemical treatment must be evaluated and considered in the light of

its limitations and proper precautions must be taken when applying it to the crop. Currently there are no fungicides registered as seed treatments for cowpea in South Africa. The results presented may aid in the development and registration of such a seed treatment. Many of the economically important diseases of cowpea are seed-borne and should be controlled to prevent any further spread of the disease and to improve the grain yield and quality. Currently there are also no fungicides registered as foliar applications for the control of cowpea diseases in South Africa. However, the foliar control of diseases, may aid in reducing the amount of inoculum available for dissemination.

The results of the studies presented in this thesis provided new and relevant information on a new cowpea disease caused by *A. cassiae*. It expanded the knowledge of cowpea pathology in South Africa and opened many doors for further research.

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PUBLICATIONS AND

PRESENTATIONS

The following publication and presentations stemmed from this study.

Publications

LA GRANGE, N. & AVELING, T.A.S. 1998. **First report of** *Alternaria cassiae* **on cowpea.** *Plant Disease* 82(10):1171.

Presentations

LA GRANGE, N. & AVELING, T.A.S. **Conidial types of** *Alternaria cassiae.* Microscopy Society of Southern Africa, 37th Congress, Johannesburg, South Africa. December 1998. Poster

LA GRANGE, N., AVELING, T.A.S. & VENTER, S.L. **A new disease of cowpea caused by** *Alternaria cassiae.* Southern African Society of Plant Pathology, 37th Congress, Pietermaritzburg, South Africa January 1999. Poster.

LA GRANGE, N. AVELING, T.A.S. & VENTER, S.L **A new disease of cowpea caused by** *Alternaria cassiae.* International World Cowpea Conference, Nigeria, September 2000. Poster.

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VAN DEN BERG, N. & AVELING, T.A.S. **Infection of** *Alternaria cassiae* **on cowpea.** Microscopy Society of Southern Africa, 39th Congress, Grahamstown, December 2000. Paper.

VAN DEN BERG, N. & AVELING, T.A.S. **Evaluation of six chemical fungicides for the control of** *Alternaria cassiae* **on cowpea.** Southern African Society of Plant Pathology, 39th Congress, White River, South Africa, January 2001. Poster.