ERGOT OF NUT SEDGE IN SOUTH AFRICA

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DECLARATION

I, the undersigned, declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy to the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Ella Johanna van der Linde

July 2005

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ERGOT OF NUT SEDGE IN SOUTH AFRICA

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RESUMÉ

Several cases of bovine ergotism ascribed to the intake of fodder contaminated with yellow nut sedge (*Cyperus esculentus*) ergotised by *Claviceps cyperi* have been reported since 1996 from the eastern Highveld region in South Africa. These were the first incidents of ergotism associated with a *Claviceps* species infecting a non-poaceous host. *Claviceps cyperi* was described in 1967 from herbarium specimens collected between 1940 and 1944 in and around Pretoria in the former Transvaal Province, South Africa, and has not been recorded elsewhere in the world. Besides the above taxonomic account of *C. cyperi* and its apparent noxiousness, no information is available on the fungus. This study was undertaken to elucidate, in part at least, the symptomology and epidemiology of the disease, and the pathology, toxicology and phylogenetic relationship of *C. cyperi*.

Symptoms of ergot on nut sedge, germination of sclerotia of *C. cyperi*, and the morphology of live specimens of the pathogen were described for the first time. Honeydew associated with the disease is inconspicuous and the initial symptom of infection was a black sooty layer on inflorescences of infected plants due to colonisation of the honeydew by the saprophytic fungus *Cladosporium cladosporioides*. Ergot sclerotia started to develop in March and April and could be discerned as small protuberances on inflorescences in the place of seed. Mature sclerotia were purplish-

black and required a resting period of about two months before germinating. Germination occurred without prior cold treatment, though exposure of the sclerotia to 5 °C for 21 days significantly increased the germination rate. Dimensions of sclerotia, stipes, capitula, asci and ascospores of live specimens were somewhat larger than in the original description, but the general morphology supported treatment of *C. cyperi* as a distinct species. Comparison of *C. cyperi* with 15 other *Claviceps* species available in the GenBank sequence database by means of multilocus PCR fingerprinting of genomic DNA and sequence analysis of the ITS1-5.8 rDNA-ITS2 and ß-tubulin gene intron 3 regions confirmed that it is a separate species, phylogenetically the closest related to *Claviceps zizaniae*, the ergot fungus of wild rice (*Zizania* spp.).

The sphacelial state of *C. cyperi* was isolated and grown in culture on various media at different temperatures. Optimal growth occurred at 24 °C, with no growth evident at 5 °C and 32 °C. The anamorph conformed to the description of *Sphacelia*, but an enteroblastic mode of conidiogenesis could not be confirmed and placement of the species in *Sphacelia* is therefore *nomen provisorium*.

Infection of yellow nut sedge by *C. cyperi* could not be achieved in the greenhouse. Microscopic examination of material collected in the field indicated that infection by *C. cyperi*, unlike most other ergot species, not necessarily mimics the pollination process, as infection of ovaries in some florets seemed to have already occurred when stylodia only started protruding. The dark layer of the omnipresent *C. cladosporioides* covering the honeydew appeared to cause a physical barrier preventing florets from opening, hence impeding development of sclerotia. *Fusarium heterosporum* was also often present in the honeydew but did not seem to have any effect on disease development. Large numbers of spotted maize beetle (*Astylus atromaculatus*) were commonly observed visiting nut sedge inflorescences, whereas larvae of an unidentified thrips species invaded and consumed the ovaries and anthers. These insects possibly contributed to the dissemination and/or natural control of the disease.

The main ergopeptine alkaloid in sclerotia of *C. cyperi* was identified by HPLC and tandem mass spectroscopy as α-ergocryptine, with small amounts of ergosine, ergocornine and ergocrystine also present. This alkaloid profile corresponds with the

alkaloid content of the fodder implicated in the outbreaks of bovine ergotism and is typically associated with "summer syndrome" symptoms observed in affected cattle. Although α-ergocryptine is toxic to humans and animals, its brominated derivative, 2-bromo-α-ergocryptine, is a valuable drug with various pharmaceutical applications. Unfortunately, all attempts at inducing *C. cyperi* to synthesise α-ergocryptine in culture for commercial use have failed.

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1. GENERAL INTRODUCTION

Ergot refers to the infection of ovaries in the florets of cereals and grasses (*Poaceae*), sedges (*Cyperaceae*) and rushes (*Juncaceae*) by 47 described and various undescribed species of the ascomycete genus *Claviceps* (*Clavicipitaceae*, *Hypocreales*) (Pažoutová 2001). According to Brady (1962), 509 species in the *Poaceae*, 17 in the *Cyperaceae* and 4 in the *Juncaceae* have been reported as hosts, although the present number of poaceous hosts is considerably higher (S. Pažoutová, pers. com.). The disease destroys between 5 and 10 % of the seed in infected heads (Agrios 1997), but its main importance is the sclerotia which are poisonous to humans and animals due to various tetracyclic ergoline toxins, collectively referred to as ergot alkaloids, contained in them (Mantle 1977). Consumption of ergot-infested food or fodder leads to ergotism, a toxicosis characterised by psychotic delutions, nervous spasms, convulsions, gangrene, abortion, infertility and reduction in milk production (Mantle 1977). Although widespread epidemics amongst humans occurred during the Middle Ages and isolated cases are still reported from time to time (www.lfra.co.uk/eman2; European mycotoxin awareness network, October 2003), refinements in grain production and processing have now reduced the incidence of ergot poisoning in humans to an insignificant level. However, ergot poisoning continues to be of economic importance as an animal disease, with outbreaks regularly being described in swine, sheep, horses, poultry, and particularly cattle (Rottinghaus *et al.* 1993).

Bovine ergotism is characterised by an affliction of animals known as "summer syndrome". Especially cows in milk are unable to effectively dissipate heat through the skin and on hot days stand salivating and gasping for breath (Figure 1.1), seek shade, lie down in puddles or mud, and even wade into dams in order to cool off (Figure 1.2). In cool weather these signs may, however, not be evident. The development of a winter hair coat also becomes evident, especially in black parts which then turn brown (Figure 1.3). With excessive intoxication in winter, necrosis of extremities such as the ear, tail and feet may occur (Claassen 1995; Schneider *et al.* 1996). Another serious complication of summer syndrome is a precipitous drop in the level of the pituitary lactogenic hormone,

prolactin, resulting in a marked drop in milk production. Conception is also seriously affected and may in the long run be of even greater economic significance than the decline in lactation. Mortalities are rare and after removal of the source of intoxication it takes about two months for milk production to return to normal and the symptoms to abate (Schneider *et al.* 1996).

The first outbreak of bovine ergotism in South Africa was reported in the late 1980's from the Western Cape Province when wild annual rye grass (*Lolium* sp.) ergotised by *Claviceps purpurea* (Fr.: Fr.) Tul. ended up in dairy rations (Claassen 1995; Schneider *et al.* 1996). During December/January 1996/97 a typical ergot syndrome was diagnosed in two Holstein dairy herds, one at Greylingstad (Mpumalanga Province) and the other in Kwazulu-Natal, between Memel and Newcastle. In the first instance, a herd of 500 Holstein cattle (240 cows, 210 in milk), were fed a total mixed ration (TMR) consisting of, amongst others, lucerne (*Medicago sativa* L.), teff (*Eragrostis tef* (Zucc.) Trotter) and *Eragrostis curvula* (Schrad.) Nees hay, and maize (*Zea mays* L.) silage. The latter was produced on the farm. Disease symptoms were typically hyperthermia, open-mouthed breathing, shade-seeking and wading into dams. Milk production was down by 33 %. (A. Lawrence, pers. com.). The maize fields utilised for silage were heavily infested with yellow nut sedge (*Cyperus eculentus* L.) (Figure 1.4) which was cut with the maize. Silage was produced in long bunkers and loads of slashed material from the lands were spread over the length of them and trampled down before spreading the next load. Due to heavy rains that particular season, fields were very wet and could not be maintained, with harvesting of the maize taking place at a very mature stage. The farm was again visited in the middle of March 1997. Cows gradually started recovering and milk production improved slowly. The reddish long hair was beginning to be shed. Only a few animals still showed symptoms of hyperthermia on hot days. In April 1997 production was returning to normal, but problems with conception persisted.

At the second farm in the Memel district, animals showed similar symptoms. Maize fields were heavily infested with yellow nut sedge. Loads of slashed maize stubble were stocked and compacted in heaps from the back to the front of the bunker and not in layers as in the first instance. The farmer fed the silage from May to August 1996. After symptoms appeared, the silage was withdrawn in the middle of September 1996, but introduced again

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in January 1997. The symptoms promptly reappeared. The farmer then withdrew the silage totally in February 1997 and by the middle of March, milk production had improved considerably. Inspection of the rations from the above two farms indicated the presence of typical ergot sclerotia in the maize silage which, upon examination of possible sources, proved to be those of a *Claviceps* species ergotising the nut sedge that infested the maize fields.

At approximately the same time milk production improved in the above case, occurrence of a typical hyperthermia syndrome was reported by a third farmer in the Vrede district. His dairy comprised 120 Holstein and 10 Jersey cows. Their TMR consisted of concentrate, maize silage and/or various mixed hays, including the farmer's own teff hay incorporated in the TMR at about 10 % until December 1996 without any problems. The teff hay component was then increased to 45 % and within about 3 weeks the summer syndrome occurred (J. Theron, pers. com.). Initially the farmer ascribed it to viral ephemeral fever (three-day-stiff sickness). However, on becoming aware of the occurrence of ergotism in the area, he suspected his teff hay as a probable cause and withdrew it from the TMR by the end of March 1997. Heavily ergotised nut sedge was observed in the previous season's baled teff hay. The following season's maize, grain sorghum (*Sorghum bicolor* (L.) Moench.) and teff fields were also heavily infested with ergotised nut sedge. Shortly afterwards, ergotism was also reported from Frankfort (Free State Province), Bethal (Mpumalanga Province), Lydenburg (Mpumalanga Province) and Settlers (Limpopo Province) (Figure 1.5).

C. esculentus and *Cyperus rotundus* L. (purple nut sedge) have a world-wide distribution (Holm *et al.* 1991; Okoli *et al.* 1997) and are troublesome weeds in virtually all cultivated soils. The two species are also widely distributed in South Africa (Gordon-Gray 1995). Doidge (1950) reported the presence of a *Claviceps* sp. on yellow and purple nut sedge in the Pretoria area. The species was subsequently identified by Loveless (1967) as *Claviceps cyperi*, but has not been collected since from anywhere in the world. The above outbreaks and the economic implications that were involved, necessitated further investigation of the nut sedge ergot syndrome.

The main objective of this thesis was to elucidate the morphology, taxonomy and phylogeny of the pathogen, the alkaloid profile, physiology and mode of infection and some aspects pertaining to the epidemiology and ecology of the disease.

To achieve these objectives, the work focused on the following aspects:

- Studying the germination process of sclerotia of *C. cyperi* and comparing the morphological characters with other *Claviceps* species occurring on the *Cyperaceae*, and some poaceous hosts.
- Describing the cultural characteristics, morphology and conidiogenesis of the *Sphacelia* state of *C. cyperi* and assessing its taxonomic position.
- Elucidating the alkaloid profile of *C. cyperi* in relation to the disease symptoms observed in affected cattle.
- Investigating the mode of infection of *C. esculentus* by *C. cyperi*.
- Characterising *C. cyperi* molecularly and establishing its phylogenetic relationship with other *Claviceps* species.

The thesis includes various chapters, each comprising an abstract, introduction incorporating a literature review, materials and methods, results with figures and tables, and a discussion of findings. This manner of presentation was followed due to the diverse nature of the different facets and to facilitate the submission of each chapter for publication:

- Chapter 2: Symptomatology and morphology of *Claviceps cyperi* on yellow nut sedge in South Africa.
- Chapter 3: The *Sphacelia* state of *Claviceps cyperi* in culture.
- Chapter 4: Mode of infection of *Cyperus esculentus* by *Claviceps cyperi*.
- Chapter 5: Ergot alkaloids produced by *Claviceps cyperi*.
- Chapter 6: Molecular systematics of *Claviceps cyperi* and other South African *Claviceps* species.
- Chapter 7: General discussion.

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Figures 1.1-1.3 Symptoms of ergotism in Holstein cows:
1.1 Salivating with mouth open and tongue protruding (Photo: T.W. Naude).
1.2 Wading into water to cool off, suffering from hyperthermia (Photo: T.W. Naude).
1.3 Development of winter coat, black parts turning brown.
Figure 1.4 Yellow nut sedge infestation of a maize field.

Figure 1.5 Localities in South Africa (indicated by red dots) from which bovine ergotism ascribed to the intake of ergotised nut sedge have been reported since 1996.

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2. SYMPTOMATOLOGY AND MORPHOLOGY OF *CLAVICEPS CYPERI* ON YELLOW NUT SEDGE IN SOUTH AFRICA

Abstract

Symptoms of ergot on nut sedge, germination of sclerotia of the causal organism, *Claviceps cyperi*, and morphology of live specimens of the pathogen are described for the first time. The initial symptom of infection was a black sooty layer on inflorescences of infected plants due to colonisation of the ergot honeydew by *Cladosporium cladosporioides*. Sclerotia of *C. cyperi* started to develop in March and April and could be discerned as small protuberances on inflorescences in the place of seed. When mature, sclerotia were purplish-black, fusiform to cylindrical, 8—15 x 1.5 mm, and smooth. They generally remained viable for less than a year and germinated without prior cold treatment, although exposure for 21 days to 5 °C before incubation significantly increased the germination rate. Under moist conditions at 24 °C in the laboratory, germination commenced within 4 to 8 weeks. Stromata took about 12 days to mature and were sand or putty-coloured. Stipes reached a length of 10—15 mm and were 0.6—0.8 mm in diameter at maturity, but continued to elongate until they eventually dried out. Mature capitula were subglobose, greyish-orange to greyish-red, 1.5—1.8 mm in diameter, distinctly lobate with a perithecium embedded in each lobe and a collar-like appendage around the base. Perithecia were ellipsoidal, 300—360 x 130—150 μm, and asci cylindrical, 175—200 x 3—3.5 μm. Each ascus contained 8 hyaline, filiform ascospores, 75—100 x 1.0—1.5 μm in size. Conidia from honeydew were aseptate, hyaline, narrowly oblong to narrowly elliptical, 8.5—11 x 2.5—3.5 μm. Dimensions of sclerotia, stipes, capitula, asci and ascospores were larger than in the original description, which was based on preserved herbarium material. However, the general morphology supports treatment of *C. cyperi* as a distinct species.

2.1 INTRODUCTION

Ergot of yellow and purple nut sedge (*Cyperus esculentus* L. and *Cyperus. rotundus* L.) was first recorded in South Africa between 1940 and 1944 from Pretoria, Hartebeespoort, Brits and Kempton Park in the former Transvaal Province (now Gauteng and North-West Provinces, amongst others) (Doidge 1950). Seven specimens of the causal organism were deposited in the National Collection of Fungi, ARC-Plant Protection Research Institute, Pretoria, as *Claviceps* species undetermined. Based on the herbarium material, which included stromata on a few sclerotia that were artificially germinated in 1944, Loveless (1967) described the species as *Claviceps cyperi*. Only two other *Claviceps* species have been described on *Cyperaceae*, namely *Claviceps grohii* Groves on *Carex* and *Claviceps nigricans* Tul. on *Eleocharis* and *Scirpus* (Brady 1962). *C. cyperi* differed from these two species, notably in having prominently papillate capitula and straw-coloured, instead of

blackish-purple, stromata and stipes. Loveless (1967) nevertheless stressed that his identification was based on preserved material, and that the *Claviceps* sp. from *Cyperus* could have been mistaken for particularly *C. nigricans* if it proved to produce blackish purple stromata in the living state. *C. cyperi* appears to be confined to South Africa and is the only *Claviceps* species thus far described on *Cyperus*, although sclerotia similar to those of *C. cyperi* have been observed in ergotised specimens of *Cyperus latifolius* Poir. from Malawi and *Cyperus rigidifolius* Steud. from Ethiopia maintained at the International Mycological Institute in Kew. The absence of stromata and conidia in the above specimens unfortunately precludes their identification to species level.

Since the description by Loveless (1967), *C. cyperi* has fallen into oblivion. However, several cases of ergotism diagnosed in dairy herds in the eastern Highveld Region of South Africa since 1996 implicated ergotised nut sedge as a possible cause. Chemical analysis revealed the presence of ergopeptine alkaloids, particularly ∝-ergocryptine, in silage fed to the cows and inspection of the silage showed it to be extensively contaminated with ergotised yellow nut sedge (Van der Linde & Naude 2000; Naude *et al.* 2005). *C. esculentus* is a common weed species distributed throughout the world (Bendixen & Nandihalli 1987), and the finding that it could be a source of ergotism obviously warranted a reappraisal of the nut sedge ergot complex. This report describes the symptoms of the disease, germination of sclerotia, and the morphology of live specimens of the pathogen.

2.2 MATERIALS AND METHODS

Specimens of *C. cyperi* (PREM 56618—56623 and 57392—57394) were collected in March each year from 1997 to 2002 from ergotised inflorescences of *C. esculentus* at four farms in three districts of the Highveld Region, viz. Greylingstad (26.47S, 28.52E), Memel (27.29S, 29.47E) and Vrede (27.33S, 29.06E) (Table 2.1), after recording the symptoms and *in situ* morphology of the sclerotia. Sclerotia were removed from the inflorescences and maintained in clean, dry containers at room temperature.

In May of each of the above years, ca. 250 sclerotia from each of the specimens indicated in Table 2.1 were sonicated for 10 minutes in three changes of sterile distilled water (SDW),

surface-disinfested for 3 minutes in 1.75 % sodium hypochlorite, rinsed several times with SDW, and blot-dried aseptically. The sclerotia were placed on sterile sand moistened (1997—1999) or flooded (2000—2002) with SDW in 95-mm-diameter glass Petri dishes (10 sclerotia per dish). Dishes were sealed with Parafilm® and five dishes of each specimen were subjected to one of the following treatments:

1. Seven days at 5 °C.
2. Seven days at 5 °C, followed by 7 days at 18 °C.
3. Twenty-one days at 5 °C.
4. Twenty-one days at -20 °C.
5. No temperature treatment.

Following treatment, the Petri dishes with sclerotia were incubated at 24 °C under intermittent (12 hour light/dark) fluorescent illumination. Sclerotia that germinated were recorded for 16 weeks, germination being considered to have taken place when the "head" or capitulum could be clearly seen protruding from the sclerotium. Observations were made of the germination process and stromatal development. Descriptions of colours were according to Kornerup & Wanscher (1963). Measurements were made with an ocular micrometer.

Data were analysed using GenStat (2000). Treatment differences were tested by analysis of variance, and Fisher's protected *t*-test least significant difference was used to separate means at 5 % level of confidence (Snedecor & Cochran 1980).

2.3 RESULTS

Ergot infection of *C. esculentus* first became apparent as a black sooty layer on inflorescences of the plants (Figure 2.1) due to colonisation by *Cladosporium cladosporioides* (Fresen.) G.A. de Vries of the sticky, sugary honeydew produced by *C. cyperi* on young inflorescences early in the growing season. *Cerebella andropogonis* Ces. was occasionally also associated with the honeydew.

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Sclerotia started to develop during mid-summer (December—January), reaching maturity during late summer/early autumn (March—April). Each inflorescence contained up to 50 sclerotia, visible as small outgrowths or "horns" on the inflorescence in the place of seed (Figure 2.2). Young sclerotia had a light brownish lilac colour, becoming purplish-black when mature. Mature sclerotia were fusiform to cylindrical, straight to slightly curved, smooth, and measured 8—15 x 1.5 mm (Figures 2.3—2.5). Sclerotia eventually dropped to the soil, often with stamen residues still attached to one end of the sclerotium.

Surface-disinfestation of sclerotia with sodium hypochlorite resulted in them being bleached light brown or straw-coloured, but did not render them entirely free of contamination by *C. cladosporioides*. In the first three germination experiments, where sclerotia were maintained on moist sand, contamination impeded germination to such an extent that the mean germination rate of freshly-collected mature sclerotia was only 12 % (Table 2.2). Partly submerging the sclerotia in a film of water, as in the last three experiments, reduced interference from contaminants considerably and increased the germination rate to 66 %. From Table 2.2 it is also evident that sclerotia generally lost their viability within a year of collection. Nevertheless, differences between temperature treatments were highly significant ($P \leq 0.001$), hence allowing pair-wise comparison of the treatments over the various seasons. This indicated (Table 2.3) that keeping the sclerotia for 21 days at 5 °C prior to incubation at 24 °C, resulted in a higher germination rate than any of the other treatments, except for exposure for 7 days to 5 °C followed by 7 days to 18 °C. The lowest germination rate occurred in sclerotia subjected to -20 °C for 21 days.

Germination commenced within 4 to 8 weeks of incubation at 24 °C. The first sign was the swelling and cracking of the cortex around sites where pinkish-grey papillae started to protrude (Figure 2.3). After 1—2 days, the swollen apex/head became visible and eventually fully emerged as the stipe elongated. At this early stage the capitulum was not yet differentiated from the rest of the stipe, but it rapidly increased in size and after 3 days was clearly discernible (Figure 2.4). Individual perithecia, each formed in a lobe of the capitulum, could readily be distinguished after 5 days (Figure 2.5), but asci were still immature. By this time the stipe had reached a length of 8—10 mm, and was sand or putty-coloured with a

swollen base. Most of the sclerotia that germinated produced more than one stroma, the maximum observed being six.

Stromata took about 12 days to mature, after which entire asci could be seen protruding through the greyish-ruby ostioles of the perithecia (Figure 2.6). Mature capitula were subglobose, greyish-orange to greyish-red, 1.5—1.8 mm in diameter and had a distinctly lobate, almost ranunculoid appearance with a perithecium embedded in each lobe and a collar-like appendage surrounding the base (Figures 2.6, 2.7). Stipes were about 10—15 mm long and 0.6—0.8 mm in diameter when the asci reached maturity and continued elongating until they eventually dried out. Perithecia were ellipsoidal, 300—360 x 130—150 µm and the asci cylindrical, 175—200 x 3—3.5 µm. Each ascus contained 8 hyaline, filiform ascospores, 75—100 x 1—1.5 µm in size (Figure 2.8).

Conidia examined directly from the honeydew were aseptate, hyaline, with their shape varying from reniform to oval to narrowly oblong or narrowly elliptical, 8.5—11.0 x 2.5—3.5 µm.

2.4 DISCUSSION

The morphology of live specimens of *C. cyperi* corresponded with the original description by Loveless (1967), particularly regarding the prominently papillate capitula with perithecia projecting well above the surface and collar surrounding the base, as well as the shape of the sclerotia, stromata, perithecia and asci. The dimensions of most structures, however, were notably larger, e.g. sclerotia 8—15 mm x 1.5 mm versus 5—8 x *ca.* 1.0 mm, stipes 10—>15 x 0.6—0.8 mm versus up to 6.0 x 0.2—0.4 mm, capitula 1.5—1.8 mm versus ≤ 1.0 mm, asci 175—200 x 3—3.5 µm versus 90—120 x 2.5—3.5 µm, and ascospores 75—100 x 1.0—1.5 µm versus 70—80 x 1.0 µm. Contrary to the above, conidia and perithecia of specimens in the present study did not differ significantly in size from the original description, viz. (5.7) 8—10 (13.2) x 2.2—3.7 µm compared to (5.5) 8.0—10.5 (13) x 2.0—3.5 (4.0) µm and 300—360 x 130—150 µm compared to 280—360 x 120—144 µm, respectively. The consistency of the perithecial dimensions is in accordance with the view of Loveless (1964, 1967) that the size of perithecia is one of the most constant characters of mature stromata of

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Claviceps spp. and therefore a reliable taxonomic criterion, whereas differences in size of the other components were expected as fungal structures are inclined to shrink due to desiccation when preserved in a herbarium. The colour of the different stromatal elements observed in this study, viz. sand or putty-coloured stipes, greyish-orange to greyish-red capitula and greyish-ruby ostioles of the perithecia, as well as elucidation of the germination process, augment the original description of *C. cyperi*.

Results of the present study support the separation of *C. cyperi* from *C. grohii* and *C. nigricans* by Loveless (1967), despite the greater dimensions of the reproductive components. In the original description of *C. nigricans*, Tulasne (1853) did not provide measurements for perithecia, asci or ascospores and distinguished the species mainly on the blackish-purple colour of the stromata, which *per se* is sufficient to separate it from *C. cyperi*. Furthermore, the illustration of the capitulum of *C. nigricans* in Tulasne (1853) bears little resemblance to that of *C. cyperi* in having neither a papillate appearance nor a collar surrounding its base. Unfortunately a viable reference specimen of *C. nigricans* could not be obtained to compare with *C. cyperi*. The single specimen of *C. nigricans* preserved in the National Collection of Fungi in Pretoria (PREM 23108, collected in 1928 from *Isolepis costata* Hochst ex A. Rich. (as *Scirpus* cf. *costatus* (A. Rich.) Boeck) in the Eastern Cape) contained only sclerotia which also failed to germinate.

According to the present description, *C. cyperi* resembles *C. grohii* more closely than *C. nigricans*, particularly regarding general appearance, orangy colour of the capitulum, and dimensions of most stromatal elements (Groves 1943). However, the blackish-violet stipes and shorter perithecia and asci of *C. grohii* (150—300 µm versus 300—600 µm and 125—160 µm versus 175—200 µm, respectively), clearly separate the two species. Comparison of *C. cyperi* and *C. grohii* by means of multilocus fingerprinting, as well as sequencing of internal transcribed spacers 1 and 2 and the 5.8S and β-tubulin intron 3 regions (Chapter 6), confirmed this separation.

Claviceps species have traditionally been regarded as having monogeneric host ranges, but it is now accepted that some can infect different host genera, even in genera in different tribes (S. Pažoutová, pers. com.). Indeed, *Claviceps purpurea* (Fr.:Fr.) Tul., which occurs on

various grain and grass species, is known to occasionally infect sedges as well (Pažoutová *et al.* 2000). Considering the sporadic occurrence of *C. cyperi* (it has not been collected from anywhere in the world since the original record in the early 1940's until the present outbreaks of ergotism) and its apparent confinement to South Africa on an exotic but nonetheless ubiquitous weed, the possibility therefore existed that the *Claviceps* on nut sedge could be a species occurring on a different host in South Africa. Besides *C. cyperi*, only five described *Claviceps* species have been recorded in the country, viz. *C. purpurea*, *Claviceps africana* Freder., P.G. Mantle & W.A.J. de Milliano, *Claviceps cynodontis* Langdon, *Claviceps digitariae* Hansf. and *Claviceps paspali* F. Stevens & J.G. Hall (Crous *et al.* 2000). Of these, *C. paspali* and *C. purpurea* were observed on *Paspalum* and *Lolium* spp., respectively, in holding camps on the farms concerned, but proved to be molecularly distinct from *C. cyperi* (Chapter 6). *C. africana* also showed no molecular relatedness to *C. cyperi*, but the relationship with *C. cynodontis* and *C. digitariae* still has to be established, particularly since hosts of these two species commonly occur on the Highveld as well as in the Pretoria area (Acocks 1988).

The most conspicuous morphological feature of *C. cyperi* is its prominently papillate capitulum. This characteristic is shared with *Claviceps ranunculoides* A. Möller described from *Setaria* in Brazil (Möller 1901) and subsequently also reported from New Mexico (Alderman *et al.* 2004), and indicates an obvious relationship between these two species. *Setaria* species occur throughout South Africa (Acocks 1988) and unidentified specimens of *Claviceps* on *Setaria sphacelata* (Schumach.) Moss have been deposited in the National Collection of Fungi in Pretoria (PREM 32196) (Doidge 1950). Inspection of mature sclerotia in these specimens indicated that they are much more curved, shorter and thicker (5—8 x 1.6—1.8 mm versus 8—15 x 1.5 mm) than those of *C. cyperi*, and brown instead of purplish-black in colour. According to the description of *C. ranunculoides* by Möller (1901), it also has longer perithecia than *C. cyperi* (400—420 µm compared to 300—360 µm). Comparison of the results of the molecular study in Chapter 6 with those of Sullivan *et al.* (2001) indicates that *C. cyperi* and *C. ranunculoides* are not conspecific.

Lastly, the duration of the resting period, temperature and moisture requirements for germination, and longevity of sclerotia, differ between ergot species and are usually

accordant with the life cycle and environmental preferences of the particular host (Mitchell & Cook 1968). Having an invasive hardy perennial weed as host, it was not expected that *C. cyperi* would have specific requirements regarding sclerotial survival and germination. Sclerotia appeared to have a relatively short resting period and an average life-span. Like all *Claviceps* spp., they required moisture for germination (Fyles 1915; Pantidou 1959). When germinated in the dark (data not presented), sclerotia developed stromata, but these did not differentiate into stipes and capitula, and hence did not produce inoculum (ascospores) for infection. Dependence on light for stromatal differentiation is probably common in *Claviceps* but has thus far been reported only for a few species (Eleuterius & Meyers 1974). Intolerance of the sclerotia to –20 °C indicates that *C. cyperi* is not adapted to xeric conditions (Langdon 1954; Pažoutová 2001).

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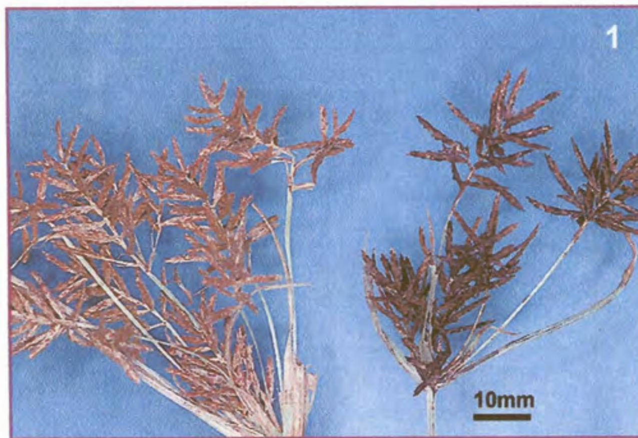


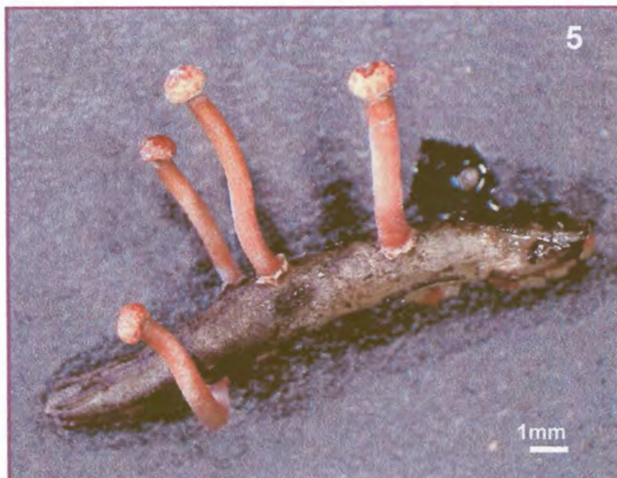
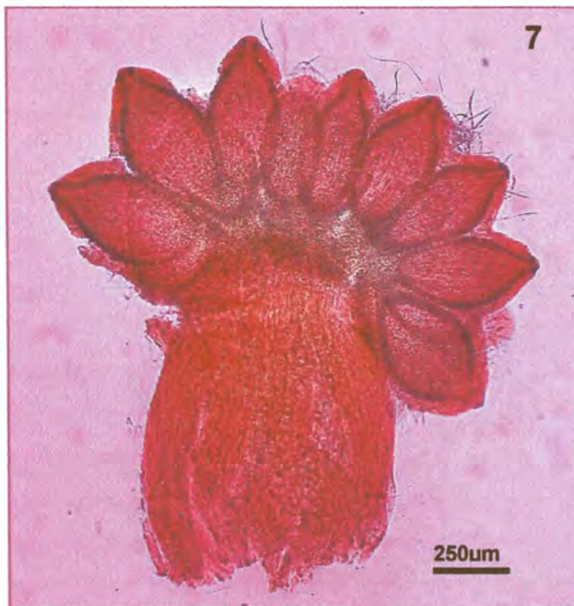


Figure 2.1	*Healthy and ergotised inflorescences of Cyperus esculentus.*
Figure 2.2	*Cyperus esculentus* inflorescences containing sclerotia of *Claviceps cyperi*.
Figures 2.3-2.6	Germination process of sclerotia of *Claviceps cyperi*.
2.3	First stage of germination of sclerotium.
2.4	Two stromata emerging.
2.5	Four stromata with stipes elongated and capitula almost mature.
2.6	Mature capitulum with individual perithecia visible – asci protruding through ostioles (arrow).



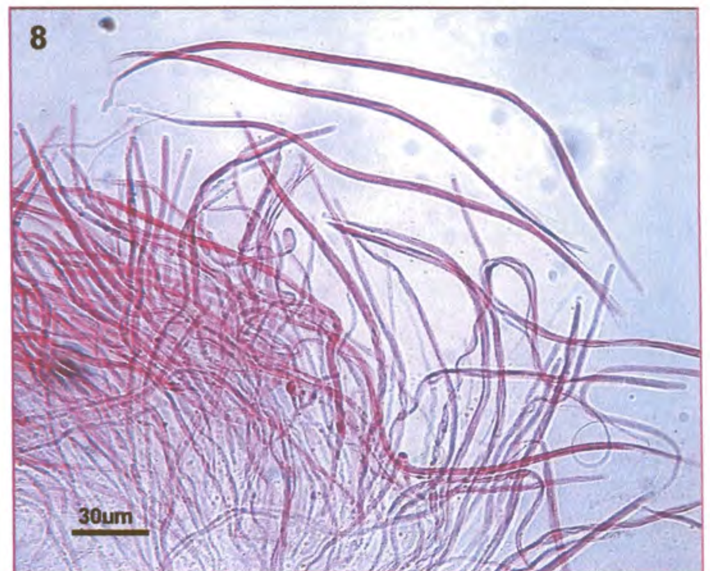


Figure 2.7 Hand-cut section through capitulum showing perithecia of *Claviceps cyperi*.
Figure 2.8 Asci of *Claviceps cyperi* with filiform ascospores.

Table 2.1 Origin of *Claviceps cyperi* specimens from *Cyperus esculentus* included in the study.

PREM	Farm	District	Collection date	Years included in germination experiments
56618	1	Greylingstad	March 1997	1997/98/99/2000
56619	2	Memel	March 1997	1997/98/99/2000
56620	3	Vrede	March 1997	1997/98/99/2000
56621	1	Greylingstad	March 1998	1998/99/2000
56622	3	Vrede	March 1998	1998/99/2000
56623	4	Greylingstad	March 1999	1999/2000
57392	1	Greylingstad	March 2000	2000
57393	1	Greylingstad	March 2001	2001
57394	1	Greylingstad	March 2002	2002

Table 2.2 Mean overall germination percentage of sclerotia of *Claviceps cyperi*.

PREM	Year collected	Germination percentage (%)					
		1997	1998	1999	2000	2001	2002
56618	1997	10.0	1.2	0.8	0.8	-[a]	-
56619	1997	8.0	0.4	0	0	-	-
56620	1997	8.1	0	0	0	-	-
56621	1998	-	16.0	0.8	0	-	-
56622	1998	-	12.8	0.4	0	-	-
56623	1999	-	-	16.8	0	-	-
57392	2000	-	-	-	64.2	-	-
57393	2001	-	-	-	-	65.4	-
57394	2002	-	-	-	-	-	66.8

[a]Not determined.

Table 2.3 Effect of temperature treatment on the germination of sclerotia of *Claviceps cyperi*.

Treatment no.	Temperature treatment	Germination percentage (%)[a]
1	7 days at 5 °C	42.8 b
2	7 days at 5 °C + 7 days at 18 °C	45.5 ab
3	21 days at 5 °C	51.3 a
4	21 days at -20 °C	20.5 c
5	None	41.5 b

[a]Germination was recorded for 16 weeks at 24 °C following treatment; each value is the mean of 24x5 replicates, each comprising 10 sclerotia; values followed by the same letter do not differ significantly according to Fischer's protected *t*-test least significant difference ($P \leq 0.05$).

3. THE *SPHACELIA* STATE OF *CLAVICEPS CYPERI* IN CULTURE

Abstract

The *Sphacelia* state of *Claviceps cyperi* was isolated and grown in culture on various media at different temperatures. Overall, best growth occurred at 24 °C, with no growth evident at 5 °C and 32 °C. Morphological features and colony appearance were the most consistent on potato-dextrose agar (PDA) at 18 °C, whereas conidiogenesis could best be observed on PDA or glucose yeast extract at 10 °C. The morphology of the nut sedge ergot anamorph is described and illustrated for the first time.

3.1 INTRODUCTION

Léveillé erected the genus *Sphacelia* in 1827 (Pantidou 1959), with *Sphacelia segetum* as type species, to describe the fungus in honeydew of ergotised grasses, aptly deriving the name *Sphacelia* from the Greek word *sphakelos* meaning "gangrene" to indicate its association with ergotism. The connection between *Sphacelia* and *Claviceps* was established by Tulasne (1853). Presently, 31 *Sphacelia* species have been validly described (www.nt.ars-grin.gov/fungaldatabases/). However, only two of these, *S. segetum* and *Sphacelia sorghi* McRae, are ergot species, the remainder being anamorphs of other genera in the *Clavicipitaceae*, e.g. *Epichloë, Balansia* and *Atkinsonella* (Rykard *et al.* 1984). All *Claviceps* species nevertheless produce a sphacelial state (Mower *et al.* 1973) and descriptions of these states are mostly based on the size and shape of conidia contained in the honeydew associated with ergot infection. The conidial morphology of many of these unnamed *Sphacelia* species is sufficiently distinct to facilitate their separation (Loveless 1964).

Although supplementary rather than essential for the identification of an ergot species, the conidial phase fulfils a vital epidemiological function, particularly as source of secondary infections, which are the most important means of spread of the disease (Loveless 1964). Since the *Sphacelia* state, unlike its *Claviceps* state, can be cultured artificially, conidial isolates are obviously also more appropriate for physiological studies such as elucidation of the chemical and physical requirements for growth and functioning

of the species, and investigation of alkaloid biosynthesis under controlled conditions. They further are more amenable to extraction of DNA for molecular analyses (Pažoutová 2001) and, being free of common honeydew contaminants like *Cladosporium, Fusarium* and yeasts (Loveless 1964), less prone to misinterpretations when describing conidial morphology.

Recent outbreaks of bovine ergotism in South Africa ascribed to the intake of fodder contaminated with yellow nut sedge (*Cyperus esculentus* L.) ergotised by *Claviceps cyperi* Loveless (Van der Linde & Naude 2000; Naude *et al.* 2005), have rekindled interest in this almost forgotten ergot species. The previous chapter provided a rediscription of the teleomorph. This report describes the isolation, culturing and morphology of the *Sphacelia* anamorph.

3.2 MATERIALS AND METHODS

Sclerotia of *C. cyperi* (PREM 57392, collected in 2000 from Greylingstad) were sonicated for 10 minutes in three changes of sterile distilled water (SDW) to rid them of superficial contaminants and dirt particles. They were then surface-disinfested in 1.75 % sodium hypochlorite for three minutes and rinsed several times in SDW.

After surface-disinfestation, the sclerotia, now soft but firm, were placed on a sterile microscope slide and studied with a dissecting microscope. The outer black wall of each sclerotium was removed with a sterile surgical blade and pinsette (Bonns 1922), and the tissue inside divided into three or four sections which were plated on glucose yeast extract agar (Fuentes *et al.* 1964). Plates were sealed with Parafilm® to prevent them from drying out and incubated at 24 °C.

Inoculum obtained from small colonies (10 mm diam.) forming from the sclerotial segments after 4 weeks were used to inoculate the following growth media, all sterilised by autoclaving for 15 minutes at 121 °C:

Potato carrot agar (PCA)

Grated potatoes	20 g
Grated carrots	20 g
pH	6.2—6.5

Boil in 1 l distilled water until soft, strain through cheesecloth and retain liquid, replenish distilled water to make up to 1 liter, add 20 g agar (Biolab).

Malt extract agar (MEA)

Malt extract (Oxoid)	15 g
Agar	20 g
pH	6.5

Potato-dextrose agar (PDA)

Potato-dextrose agar (Biolab)	39 g
pH	6.3

Corn meal agar (CMA)

Corn meal agar (Oxoid)	17 g
pH	6.4

Glucose yeast extract agar (GYA)

Glucose	10 g
Yeast extract	10 g
Agar	15 g
pH	6.2

Ten plates of each medium were incubated in the dark at 5, 10, 18, 24 and 32 °C, respectively. Colony diameters were measured at right angles after 7, 14, 21 and 28 days, and the daily growth rates calculated. Colony- and morphological characteristics of the fungus were also observed on the above media, as well as in cultures incubated under 12 hour intermittent near-UV illumination (320—420 nm).

The entire experiment was repeated with an isolate obtained from sclerotia of *C. cyperi* PREM 57394, collected in 2003 from Greylingstad. The two *Sphacelia* isolates were deposited in the live culture collection of the National Collection of Fungi in Pretoria as PPRI 7175 and PPRI 7176 respectively, where they are maintained under water and oil, as well as in lyophilised state.

Material for scanning electron microscopy was obtained from cultures of PPRI 7175 and PPRI 7176 grown on PDA at 24 °C. Blocks of agar (5 mm x 5 mm) were cut from the colonies and fixed in 2.5 % glutaraldehyde in 0.075 M phosphate buffer (pH 7.4—7.6) for at least one hour. The material was then rinsed three times for 5 minutes each in 0.075 M phosphate buffer, fixed overnight in 0.25 % aqueous osmium tetroxide overnight, and rinsed three times with distilled water. Samples were dehydrated in ethanol (70 %, 3 x 100 %), critical point dried in liquid CO_2, mounted on stubs, sputtered with gold (Roux *et al.* 2000), and examined with a Jeol 840 scanning electron microscope at 5 kV. Material for transmission electron microscopy was fixed and dehydrated in the same manner, infiltrated with 30 and 60% Quetol in acetone, embedded in 100 % Quetol and polymerised at 65 °C for 24 hours. Sections (0.5 μm thick) were contrasted in 4 % aqueous uranyl acetate for 10 minutes followed by 2 minutes in Reynold's lead citrate, and stained with Toluidine blue (Roux *et al.* 2000). Sections were examined at 5 kV with a Philips EM 301 transmission electron microscope.

Data were analysed using the statistical program GenStat (2000). A combined ANOVA was performed on the two sets of data to test for differences between treatments and the temperature-by-treatment (TEMP.TMT) interaction. Data were acceptably normal with homogenous treatment variances. Treatment means were separated using Fisher's protected *t*-test least significance difference at 5 % level of significance (Snedecor & Cochran 1980), if the F-probability from the ANOVA was significant at 5 %.

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3.3 RESULTS

No growth was evident at 5 °C or 32 °C. Overall, radial growth rate was significantly the highest at 24 °C, second-highest at 18 °C, and slowest at 10 °C (Table 3.1). Growth rate also increased with time at the two higher temperatures, but remained constant and even declined somewhat at 10 °C. Temperature affected the rate of growth on the various media differently. Best growth at 18 °C occurred on PCA. PCA also supported good growth at 24 °C, of the same order as CMA on which the fungus grew the fastest. However, at 18 °C CMA sustained the slowest growth, whereas growth at 10 °C was the slowest on PCA, albeit not significantly slower than on PDA. Cultures on all media had a peculiar yeast-like odour.

Cultures incubated at 24 °C sporulated poorly or not at all and formed bundles of hyphae (funicles). Isolates also tended to produce less conidia after three to four transfers on agar, regardless of medium or incubation temperature. Sporulation was more abundant at 18 °C than at 10 °C, complicating observation of conidiogenous structures at the former temperature. Conidiogenesis could best be observed in cultures incubated at 10 °C on either GYA or PDA, though morphological characteristics and colony appearance were the most consistent on PDA at 18 °C. The following description of the *Sphacelia* phase of *C. cyperi* pertains to the latter medium and temperature: Colonies about 12 mm in diameter after 14 days, velutinous, white, smooth to rugose (Figure 3.1), reverse light brown (Kornerup & Wanscher 1963). Hyphae 1—1.5 μm in diameter, densely compacted into masses that tear rather than unravel. Enlarged cells, either intercalary or terminally, present in some hyphae (Figure 3.2). Conidiophores absent. Conidia hyaline, aseptate, varying in shape from ellipsoidal, bacilliform, clavate to reniform, but predominantly asymmetrical with rounded tips (rarely acute) and obtuse bases, (6)10—14(17) μm long and (2.5)3.0—3.5(5.0) μm wide (Figure 3.3).

Conidiogenesis observed with both SEM (Figures 3.4, 3.5) and TEM (Figure 6) appeared to be holoblastic with conidia maturing by diffuse wall-building, secession being schizolytic

(Figure 3.6) without further proliferation of conidiogenous cells. Conidiation *in plantae* was not studied.

3.4 DISCUSSION

No study comparing the temperature and substrate preferences of a *Claviceps* anamorph has previously been published, probably because such attempts, if any, have provided the same conflicting results as the present investigation. It is nevertheless clear from the results that the *Sphacelia* state of the nut sedge ergot fungus is strictly mesophilic, not adapted to tropical or xeric environments, as also indicated by the intolerance of sclerotia of *C. cyperi* to sub-zero temperatures (Chapter 2) and the poor or no growth of cultures in liquid medium at 15 and 34 °C (Chapter 5). The results also showed that conditions optimal for vegetative growth of the fungus are not the best suited for studying conidiogenesis.

The colony morphology of the *Sphacelia* state of *C. cyperi* was essentially the same as that reported for other *Claviceps* species grown in culture. Bonns (1922) described colonies of *Claviceps purpurea* (Fr.:Fr.) Tul. as having no gross mycelial characters developing, resembling in general appearance some actinomycetes in culture. McCrea (1931) referred to cultures of *C. purpurea* as being gelatinous, spreading slowly in all directions from the point of inoculation. Colonies of *Claviceps gigantea* S.F. Fuentes, Isla, Ullstrup & Rodriquez showed a marked folding of the surface, with the mycelium compact, felty and white (Fuentes *et al.* 1964). This folding was also evident in cultures of *C. cyperi* produced directly from sclerotial tissue. Once subcultured, colonies tended to be smoother and flat on the agar surface (cf. Chapter 4). Colonies of *C. cyperi* had a light brown reverse and yeast-like odour. The enlarged cells present in some of the hyphae probably were sclerotial primordia. McCrea (1931) found that partially sclerotoid mycelium of *C. purpurea* was readily obtained on standard medium, though such tissue did not develop the morphological characteristics of true sclerotia. Cultures incubated under near-UV illumination did not differ in morphology from those incubated in the dark (data not presented).

Conidia of *Sphacelia* species vary considerably in size and shape, but are all hyaline and usually aseptate. Conidial shape has mostly been described as ellipsoidal, e.g. *C. purpurea*

(Bonns 1922; McCrea 1931), *Claviceps maximensis* T. Theis (Theis 1952) and *Claviceps digitariae* Hansf. (Herd & Loveless 1965), though some species produce allantoid, falcate, oblong, reniform and even triangular conidia (Loveless 1964). Conidial size varies from 2.2—8.5 x 1.3—3.2 µm in *C. purpurea* (McCrea 1931) to 10—30 x 5—11 µm in *C. maximensis* (Theis 1952). Loveless (1964) proposed a system based on conidial characters according to which ergot fungi could be classified into 13 groups. Conidia of *C. cyperi* varied in shape but were mostly ellipsoidal to reniform. According to these shapes and the conidial sizes observed in this study, the species best fits into group 5 of Loveless (1964). However, conidia in herbarium material and honeydew (Loveless 1967; Chapter 2) were notably smaller, probably due to desiccation. Based on their dimensions, *C. cyperi* would be classified into group 3.

According to the characteristics observed in the present study and in Chapter 3, the anamorph of *C. cyperi* conforms to the description of *Sphacelia* by Saccardo (1886). With the advent of conidial ontogeny, conidiogenesis in the *Clavicipitaceae* has been described as enteroblastic or enteroblastic-phialidic (Von Arx 1981; Rykard *et al*. 1984; White 1997), although some genera in the subfamily *Clavicipitoideae* (e.g. all *Balansia* spp. and the *Ephelis* conidial state of *Atkinsonella hypoxylon* (Peck) Diehl) produce conidia holoblastically. Recent studies by Pažoutová *et al.* (2004) have shown that conidiation within *Claviceps* anamorphs has not been elucidated fully and that some aspects need to be investigated further, including conflicting data about conidiation in culture and *in plantae*. According to Pažoutová *et al.* (2004), *Claviceps* has two distinct anamorphs: typical enteroblastic *Sphacelia* forms, and an unnamed holoblastic type related to secondary conidiation which, in some species, may be the prevalent one. TEM observations in the present study suggest that holoblastic conidiogenesis may be present in cultures of *C. cyperi*. This finding, as well as the observations by Pažoutová *et al.* (2004), could imply a revision of the genus *Sphacelia*.

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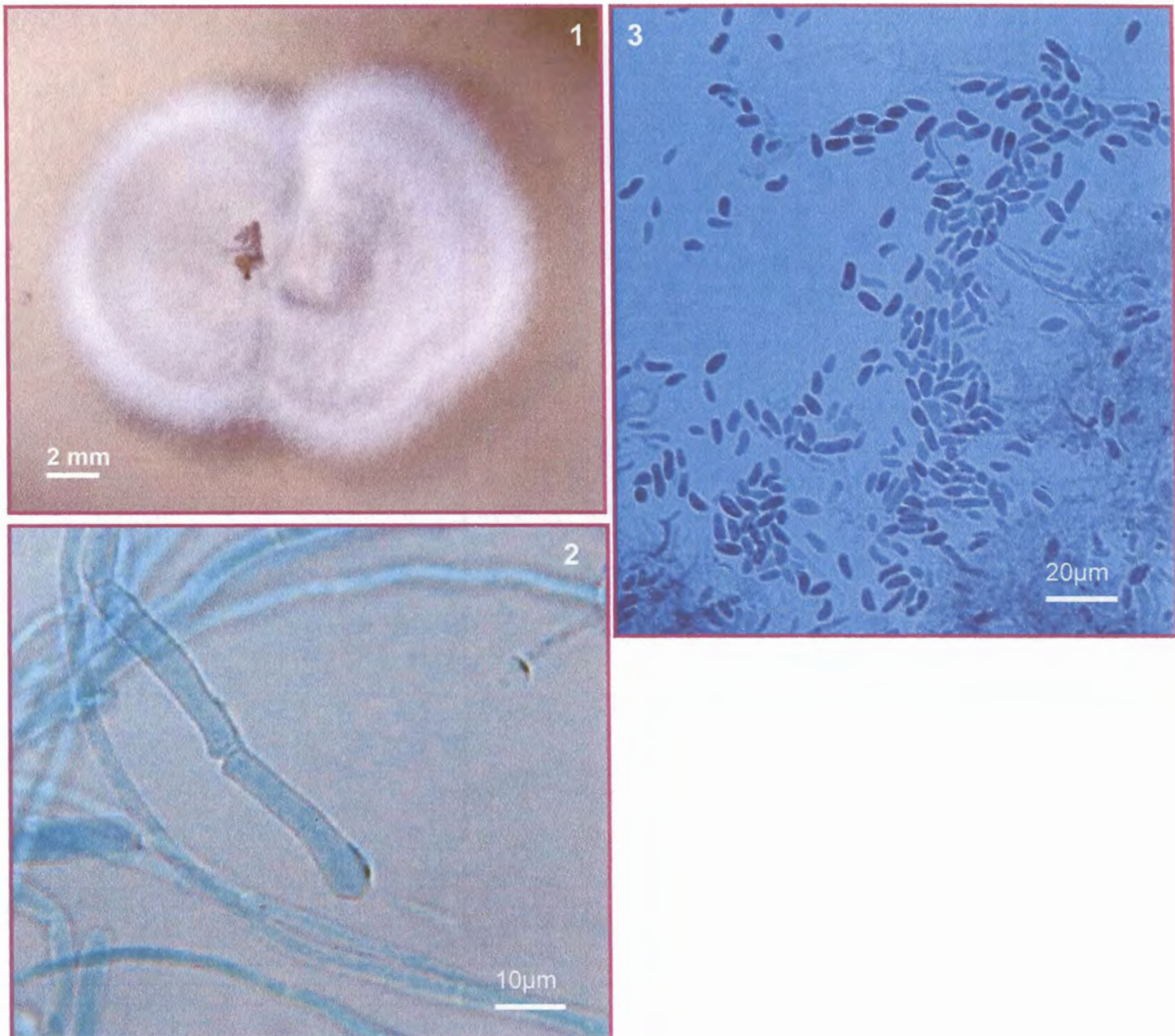
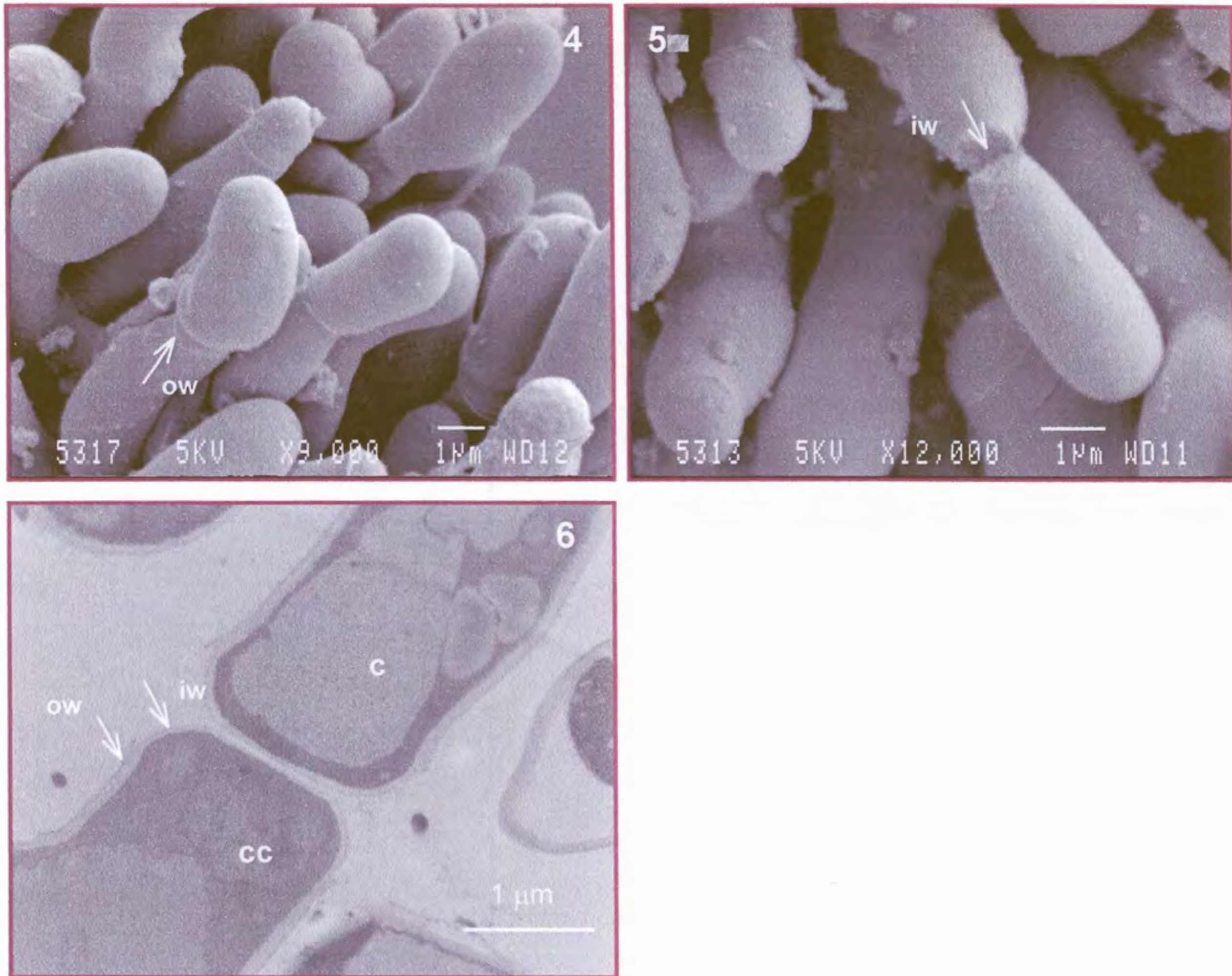


Figure 3.1 Culture of the *Sphacelia* state of *Claviceps cyperi* on potato-dextrose agar after 2 weeks.
Figure 3.2 Enlarged cells observed in hyphae of the *Sphacelia* state of *Claviceps cyperi*.
Figure 3.3 Conidia of the *Sphacelia* state of *Claviceps cyperi* produced on 2 % malt extract agar.



Figures 3.4-3.6 Conidiogenesis in the *Sphacelia* state of *Claviceps cyperi*:
3.4 Conidiogenous cells producing conidia (SEM micrograph).
3.5 Conidium seceding from conidiogenous cell (SEM micrograph).
3.6 Section through conidiogenous cell (cc) and conidium (c) with arrows indicating inner wall (iw) and outer wall (ow) (TEM micrograph).

Table 3.1 Growth rate of the *Sphacelia* state of *Claviceps cyperi* on different media at different temperatures.

Temperature °C	**Medium**	**Growth rate (mm day^{-1})[a]**				
		Day 7	Day 14	Day 21	Day 28	Mean
10	PCA	0.371 b	0.400 bc	0.343 d	0.371 b	0.371 d
	MEA	0.543 a	0.500 a	0.505 a	0.471 a	0.505 a
	PDA	0.457 ab	0.343 c	0.410 cd	0.400 b	0.402 cd
	CMA	0.543 a	0.429 abc	0.419 bc	0.393 b	0.446 bc
	GYA	0.486 ab	0.443 ab	0.486 ab	0.421 ab	0.459 ab
LSD[c]		0.139	0.094	0.069	0.060	0.054
18	PCA	1.143 a	1.314 a	1.286 b	1.479 b	1.305 a
	MEA	0.886 b	1.114 b	1.391 a	1.536 ab	1.232 b
	PDA	0.743 c	0.786 c	0.991 c	1.100 c	0.905 d
	CMA	0.743 c	0.843 c	0.781 d	0.879 d	0.811 e
	GYA	0.857 bc	0.843 c	1.267 b	1.550 a	1.129 c
LSD[c]		0.139	0.094	0.069	0.060	0.054
24	PCA	0.800 c	1.614 b	1.686 a	1.621 b	1.430 ab
	MEA	1.057 b	1.357 c	1.324 c	1.786 a	1.381 b
	PDA	0.771 c	0.829 e	0.924 d	1.000 d	0.881 d
	CMA	1.257 a	1.729 a	1.410 b	1.414 c	1.452 a
	GYA	0.886 c	1.000 d	1.371 bc	1.429 c	1.172 c
LSD[c]		0.139	0.094	0.069	0.060	0.054
LSD[d]		0.080	0.054	0.040	0.035	0.031

[a] Mean of 10 replicates of each of two isolates (PPRI 7175 and 7176) of *C. cyperi*; values in columns within temperatures followed by the same letter do not differ significantly according to Fisher's protected *t*-test least significant difference ($P \leq 0.05$).
[b]PCA = potato carrot agar; MEA = malt extract agar; PDA = potato-dextrose agar; CMA = corn meal agar; GYA = glucose yeast extract agar.
[c]LSD for values within temperatures.
[d]LSD for values within media.

4. MODE OF INFECTION OF *CYPERUS ESCULENTUS* BY *CLAVICEPS CYPERI*

Abstract

Florets of *Cyperus esculentus* infected with *Claviceps cyperi* were collected in the field to study the infection process. Light- and scanning electron microscopy indicated that infection by *C. cyperi*, unlike most other ergot species, not necessarily mimics the pollination process as infection of ovaries in some florets seemed to have already taken place when stylodia only started protruding. *C. cyperi* showed some *in vitro* antibiotic activity against two common honeydew-colonising fungi, *Cladosporium cladosporioides* and *Fusarium heterosporum*, but the omnipresent thick growth of *C. cladosporioides* covering ergotised florets appeared to impede sclerotial development by *C. cyperi*. Adult spotted maize beetles (*Astylus atromaculatus*), which were commonly observed visiting nut sedge inflorescences, probably contributed to the dissemination of ergot inoculum. Larvae of an unidentified thrips species were found in some of the florets could have facilitated entry of the pathogen, though they also destroyed infected ovaries and anthers, thereby reducing infection. Artificial infection of *C. esculentus* with conidial suspensions of *C. cyperi* could not be achieved in the greenhouse.

4.1 INTRODUCTION

Successful infection by ergot fungi depends on various factors involving the morphology and physiology of flowers on the host, as well as environmental variables (Campbell 1957; Bretag & Merriman 1981; Frederickson & Mantle 1988). The infection process of these fungi, be it *in vitro* or in the field, has consequently been the topic of several investigations (Fyles 1915; Lewis 1945; Ramstad & Gjerstad 1955; Campbell 1957; Jenkinson 1958; Fuentes *et al.* 1964; Futtrell & Webster 1965; Brewer & Loveless 1977; Luttrell 1977; Rykard *et al.* 1984; Frederickson & Mantle 1988). These studies provided vital information regarding the pathology, epidemiology and control of the disease. The results could also be applied to commercial production of ergot and to aspects such as the relationship between insects and infection, testing of crops bred for resistance to the disease, taxonomy of species of *Claviceps*, and the study of physiological specialisation within a species (Lewis 1945). It is generally accepted that ergot infection mimics the pollination process (Ramstad & Gjerstad 1955; Willingale *et al.* 1986; Willingale & Mantle 1987; Frederickson &

Mantle 1988), though inconsistencies in reports on infection by different *Claviceps* species preclude extrapolating from one species to another (Luttrell 1977).

No information is available on the mode of infection of yellow nut sedge (*Cyperus esculentus* L.) by the ergot fungus *Claviceps cyperi* Loveless, recently reported to be responsible for outbreaks of bovine ergotism on the eastern Highveld, South Africa (Van der Linde & Naude 2000). Several attempts, adhering to established procedures (Lewis 1945; Theis 1952; Campbell 1957; Brewer & Loveless 1977; Frederickson & Mantle 1988), were consequently made to artificially infect *C. esculentus* by spraying florets at all stages of development with a conidial suspension of *C. cyperi* in the greenhouse. These attempts, however, invariably failed. Inflorescences of *C. esculentus* were then collected in the field for studying the process and progress of infection, as well as the involvement of associated organisms. Results of this investigation are presented here.

4.2 MATERIALS AND METHODS

4.2.1 Microscopy

Florets of *C. esculentus* varying from very young (with styles not protruding) to fully developed and visibly infected (Figure 4.1) were collected during February 2002 in nut sedge-infested maize fields near Greylingstad. At the laboratory, the glumes of some florets were removed to expose the ovaries. Specimens were fixed in 2.5 % glutaraldehyde in 0.075 M phosphate buffer (pH 7.4—7.6) for one hour, rinsed three times for five minutes each in 0.075 M phosphate buffer, transferred to 0.25 % aqueous osmium tetroxide, and rinsed three times in distilled water. The fixed material was dehydrated in ethanol (70 % + 3x 100 %), critical point dried in liquid CO_2, mounted on stubs, sputtered with gold, and examined with a Jeol 840 scanning electron microscope at 5 kV. Material for light microscopy was embedded in Quetol epoxy resin and cut with a microtome into 5 μm—thick sections. The sections were stained with 0.5 % Toluidin O in 1 % borax, examined with a Leitz Laborlux D light microscope and photographed with a Leica DC 300 digital camera.

4.2.2 Honeydew-colonising fungi

As indicated in Chapter 2, honeydew produced by *C. cyperi* was consistently colonised by *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, whereas *Cerebella andropogonis* Ces. could occasionally be isolated. The latter species was absent in the present study, but a *Fusarium* species, subsequently identified as *Fusarium heterosporum* Nees, occurred quite frequently in the honeydew. Isolates of *C. cladosporioides* and *F. heterosporum* were tested in dual culture for antagonism towards *C. cyperi*. Cultures of the *Sphacelia* state of *C. cyperi* were established on potato-dextrose agar and allowed to grow for two weeks after which ten plates were each inoculated with either *C. cladosporioides* or *F. heterosporum*. Plates were incubated at 24 °C and examined regularly.

4.2.3 Associated insects

During flowering of maize, large numbers of a beetle species were commonly observed visiting inflorescences of both the nut sedge and maize. Specimens of the beetle were collected in sample bottles for identification. Larvae of a thrips species could be discerned inside many of the florets when preparing material for microscopy.

4.3 RESULTS

4.3.1 Microscopy

The styles in florets of *C. esculentus* are each divided into three stylodia (Figures 4.2, 4.3). The pistils, consisting of stigmata, stylodia and ovaries, are covered by glumes that are tightly closed when young, opening slightly to expose the ovaries, stylodia, stigmata and anthers as they mature (Figure 4.3).

Macroscopically, the first sign of infection was the sticky honeydew that appeared on the inflorescences, covered with a dark layer of *C. cladosporioides* (Figure 4.1). This layer appeared to cause a physical obstruction preventing the florets from opening, hence impeding sclerotial development by *C. cyperi*.

Infection appeared to have already taken place in some florets when stylodia only started protruding. Conidia could be observed on the stigmata and stylodia at an early stage (Figures 4.4—4.7) and often also on the ovaries (Figures 4.8—4.11). Some conidia could be seen germinating (Figure 4.5), with infection spreading to other parts of the pistils (Figures 4.10, 4.11). Stylodia seemed withered when infection was severe and spread over the bases of the stylodia and ovaries (Figures 4.10, 4.11).

A dense conidial mass interspersed with hyphae was evident in advanced stages of infection (Figures 4.12—4.15). This mass eventually formed a solid structure which differentiated into a sclerotium (Figures 4.16, 4.17). Remains of the stylodia were often visible at the apex of the sclerotia (Figure 4.17). The texture of the rind (outer wall or peridium) differed from that of the inner part of the sclerotium, the rind being more compact with smaller cells and the inner mass less compact with bundles of longer hyphae (Figures 4.18, 4.19).

Under the light microscope it did not seem as if infection started through conidia germinating on the stigmata and growing down the stylodia towards the ovaries (Figures 4.20—4.23). Stylodia mostly appeared intact with little or no infection evident (Figure 4.21), while ovaries in the same sections showed extensive infection (Figures 4.20, 4.22). Elongated hyphal cells could be seen throughout the ovaries (Figures 4.22, 4.23). A layer of conidia was present around the ovary (Figure 4.20) with "labyrinthine chambers" starting to form (Figure 4.22).

4.3.2 Honeydew-colonising fungi

No inhibition of mycelial growth of *C. cyperi* by *C. cladosporioides* or *F. heterosporum* was evident. Indeed, the ergot pathogen seemed to have a suppressive effect on the two saprophytes (Figures 4.24, 4.25), but was overgrown by them after 10 days.

4.3.3 Associated Insects

The beetles collected from infected nut sedge inflorescences were identified by Ms E. Grobbelaar of the National Collection of Insects (ARC-PPRI) as adult spotted maize

beetle (*Astylus atromaculatus* Blanchard). The thrips species could not be identified, but it was clear from the damage to the ovaries and anthers that the larvae were feeding on them.

4.4 DISCUSSION

Ergot is essentially a replacement disease (Tulasne 1853; Luttrell 1980), i.e. pollen of the host is replaced by spores of the pathogen, pollination by penetration, fertilisation by infection, and seed development by formation of sclerotia. Fertilisation usually renders flowers resistant to ergot infection (Burton & Levebvré 1948; Fuentes *et al.* 1964; Futrell & Webster 1965; Willingale *et al.* 1986), implying that viable inoculum of the pathogen has to be present immediately prior to, or at the time of, pollination. It is also obvious that a host in which the glumes do not open at all, or ones in which they are open for a short period of time, would provide only slight opportunity for infection (Campbell 1957). Although *C. cyperi* propagates mainly vegetatively (Tumbleson & Kommedahl 1961; Garg *et al.* 1967; Jansen 1971; Stoller *et al.* 1972; Mulligan & Junkins 1976; Stoller & Sweet 1987), it is a prolific self-pollinating flower producer (Brady 1962; Thullen & Keeley 1979). However, less than 5 % (0—19 %) of the flowers that are formed normally produce seed (Thullen & Keeley 1979). This semi-celibate nature of the weed was also evident from the relative paucity of *C. cyperi* sclerotia in inflorescences of *C. esculentus* in the field. Each inflorescence, comprising up to 2500 florets (Thullen & Keeley 1979), on average contained only 5—15 (maximum 35) ergot sclerotia. The dearth of susceptible target organs in florets of *C. esculentus*, together with the tendency towards delayed anthesis inherent to the species, probably was one of the reasons why artificial infection with *C. cyperi* could not be attained in the greenhouse. Direct inoculation into the florets (Thakur *et al.* 1983) or clipping away the tips of the glumes before spraying (Campbell 1957) could have been attempted but were deemed unpractical due to the small size of the florets.

As indicated above, ergot infection tends to mimic the pollination process. With species such as *Claviceps fusiformis* Loveless, *Claviceps paspali* F. Stevens & J.G. Hall and *Sphacelia sorghi* McRae, this pseudo-pollination process is the primary means of infection and direct penetration of ovaries is rare (Willingale *et al.* 1986; Willingale &

Mantle 1987; Frederickson & Mantle 1988). Direct infection of ovaries is more common in *Claviceps purpurea* (Fr.:Fr.) Tul. (Frederickson & Mantle 1988), but spore germination, penetration and hyphal growth down the stigma by this ergot species also closely follows the path taken by pollen. After germination and penetration hyphae grow between the cortical cells of the stylodial axis, and within the electron-dense matrix of the conspicuous pollen transmitting tract until they reach and enter the ovary (Willingale *et al.* 1986; Willingale & Mantle 1987). With *C. cyperi*, however, penetration of the stigmata and stylodia was not observed although extensive superficial colonisation of these organs occurred. It therefore seems if direct penetration through the ovary wall is the primary path of infection in nut sedge ergot, which is in accordance with the apparent ill-adaptedness of the host to sexual reproduction. Concerning the greater similarity in the mode of infection of *C. cyperi* to *C. purpurea* than to *C. fusiformis* or *C. paspali*, it is interesting to note that the sclerotia produced by *C. cyperi*, like those of *C. purpurea* (Luttrell 1977), are more complex than those of *C. fusiformis*.

Colonisation of ergot honeydew and sclerotia by saprophytic fungi is a common phenomenon. However, considering the abundance of nutrients in these substrates, it is surprising that the taxa reported from them are limited to species in the genera *Cerebella*, *Cladosporium*, *Epicoccum*, *Fusarium*, as well as some unidentified yeasts (Ajrekar 1926; Rhind 1928; Gonçalves 1937; Chalaud 1940; Langdon 1942; Simpson & West 1952; Theis 1952; Schol-Schwarz 1959; Loveless 1964; Mantle 1965; Futrell & Webster 1966; Cunfer 1975; Mower *et al.* 1975; Cole *et al.* 1981; Frederickson & Mantle 1988; Bandyopadhyay *et al.* 1990, 1998; Ali *et al.* 1996; Pažoutová & Kolínská 1999; Blaney *et al.* 2000). It is therefore likely that *Claviceps* species have evolved certain defence mechanisms which protect them from other fungi, as previously proposed by Mower *et al.* (1975) and evident from the *in vitro* inhibitory effect of *C. cyperi* on *C. cladosporioides* and *F. heterosporum* in the present study.

Antimycotic activity has not been reported for other *Claviceps* species, but is probably common to the genus. However, despite the presence of such or alternative defence mechanisms inherent to *Claviceps*, most reports indicate that honeydew-colonising fungi suppress the formation of ergot sclerotia, as was also observed with *C. cladosporioides* in the present study. Although *Cladosporium* species are frequently associated with

ergot (Futrell & Webster 1966; Frederickson & Mantle 1988; Bandyopadhyay *et al.* 1990), this is the first record of *C. cladosporioides* specifically from honeydew. *F. heterosporum*, on the other hand, is probably the most commonly reported *Fusarium* species from ergot (Futrell & Webster 1966; Cunfer 1975; Mower *et al.* 1975; Cole *et al.* 1981; Ali *et al.* 1996; Raybould *et al.* 1998). Indeed, in South Africa it has been recorded exclusively on ovaries of no less than 17 different poaceous hosts infected with either ergot or smut fungi (Doidge 1950). Both *C. cladosporioides* and *F. heterosporum* are mycotoxigenic species. The most important toxin produced by *C. cladosporioides* is emodin (Daunter & Greenshields 1973; Jacyno *et al.* 1993), whereas *F. heterosporum* has been reported to produce fusaric acid (Bacon *et al.* 1996) and various trichotecenes (Cole *et al.* 1981). The primary symptom associated with trichothecene toxicosis (Marasas *et al.* 1984) is feed refusal, while emodin has a diarrheagenic effect (Wells *et al.* 1975). Considering the prolific growth of *C. cladosporioides* and *F. heterosporum* on and in ergotised nut sedge honeydew, it is likely that the above mycotoxins were produced in significant quantities in the honeydew, and probably induced toxic effects additive to those of the ergot alkaloids, particularly reduced food intake and loss of body mass. Emodin furthermore has mutagenic activity (Brown & Brown 1976; Wehner *et al.* 1979), implying that prolonged intake of the compound could induce cancer. Although it has not been confirmed, there is also a possibility that emodin, like aflatoxin for instance (Raisbeck *et al.* 1991), can be excreted in a still toxic form in milk destined for human consumption.

Various moth, fly, leafhopper, thrips, beetle, ant and bee species have been implicated in the dissemination of *Claviceps* inoculum (Futrell & Webster 1966; Mower *et al.* 1975; Butler *et al.* 2001; Prom *et al.* 2003; Prom & Lopez 2004), whereas one, the ergot beetle (*Acylomus ergoti* Casey) is exclusively associated with ergot honeydew (Lewis 1945; Langdon 1952). However, no reference could be traced relating *A. atromaculatus*, of which large numbers were observed to visit nut sedge and maize inflorescences in the present study, to ergot in any other plant species. *A. atromaculatus* is native to South America and was accidentally been introduced to South Africa in 1916 (Annecke & Moran 1982). Despite being mainly a pollen feeder (Annecke & Moran 1982; Human & Nicolson 2003), it is regarded as a serious pest of several crops, including maize. Adult *A. atromaculatus* beetles forage on tassels at the tips of maize cobs and sides of

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sorghum ears, whereas larvae cause damage to seed after sowing (Drinkwater 1997). Although isolation of *C. cyperi* was not attempted from the *A. atromaculatus* specimens collected in this study, it can be assumed that the beetles carried and disseminated conidia of the pathogen. They are attracted particularly by plants with yellow flowers (Drinkwater 1997) and probably fed on the tiny stylodia sometimes protruding from nut sedge florets, in the process infecting the florets with ergot conidia adhering to them. The larvae of the unidentified thrips species could also have been a source of infection, but at the same time seemed to contribute to natural control of the disease by consuming ovaries and anthers in the nut sedge inflorescences, including those infected by *C. cyperi.* Their presence in the nut sedge florets suggests that they. The inability to achieve artificial infection in the greenhouse in the absence of this thrips species suggests that adults and/or larvae of these insects could actually be integral to the life cycle of *C. cyperi.*

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Figure 4.1 Inflorescence of *Cyperus esculentus* infected with *Claviceps cyperi*. Drops of honeydew, as well as black layers formed by *Cladosporium cladosporioides* are clearly visible.

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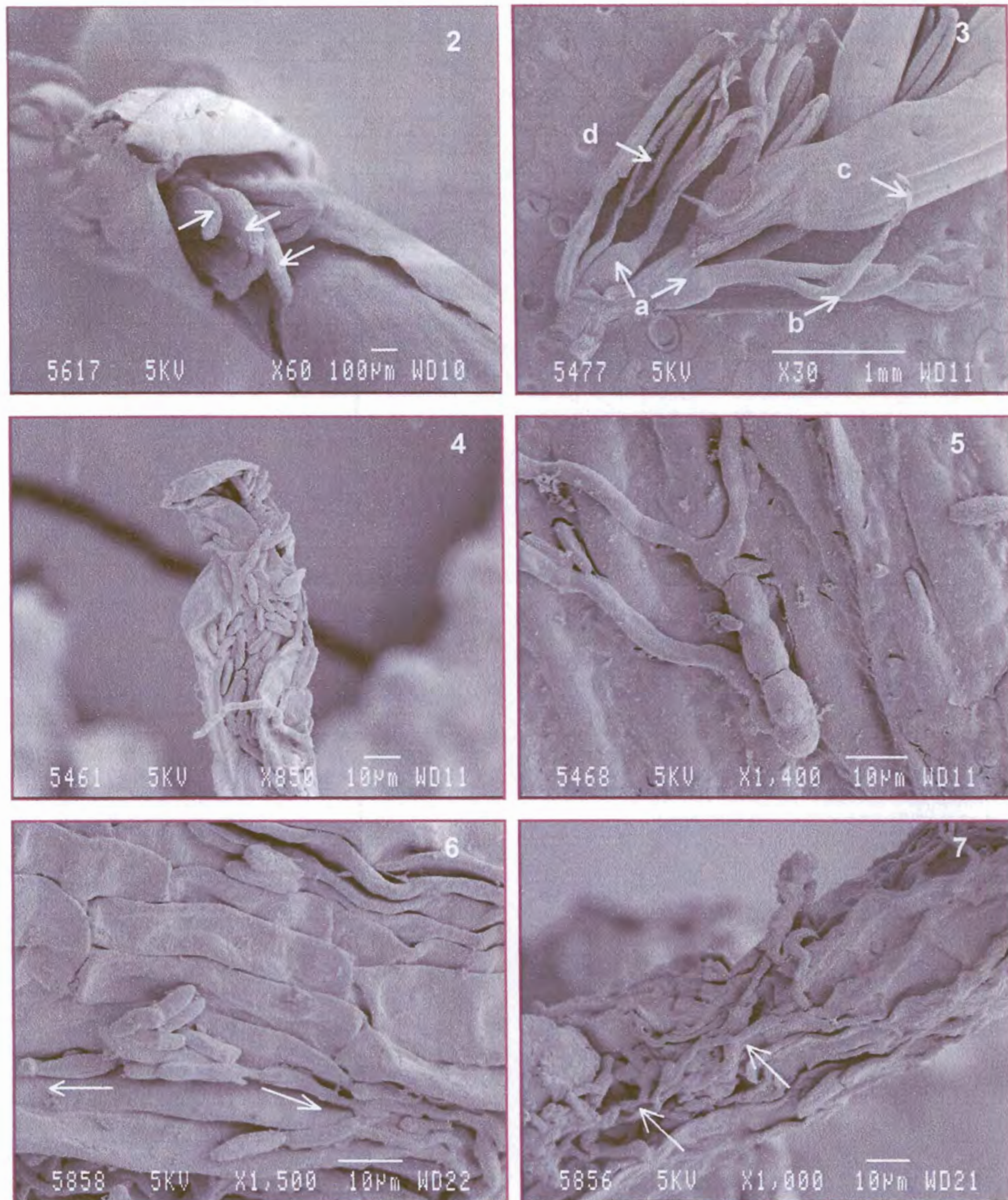

Figures 4.2-4.7 SEM micrographs of infection of *Cyperus esculentus* with *Claviceps cyperi*:

4.2 Young stylodia (arrows) protruding through glume opening.

4.3 Morphology of the pistil and stamen: (a) ovary, (b) stylodium, (c) stigma, (d) anther.

4.4 Stigma infected superficially by conidia.

4.5 Conidium forming germ tubes on the stylodium and spreading.

4.6 Mycelium spreading along length of stylodium (arrows).

4.7 Conidia and mycelium clearly visible on base of style (arrows).

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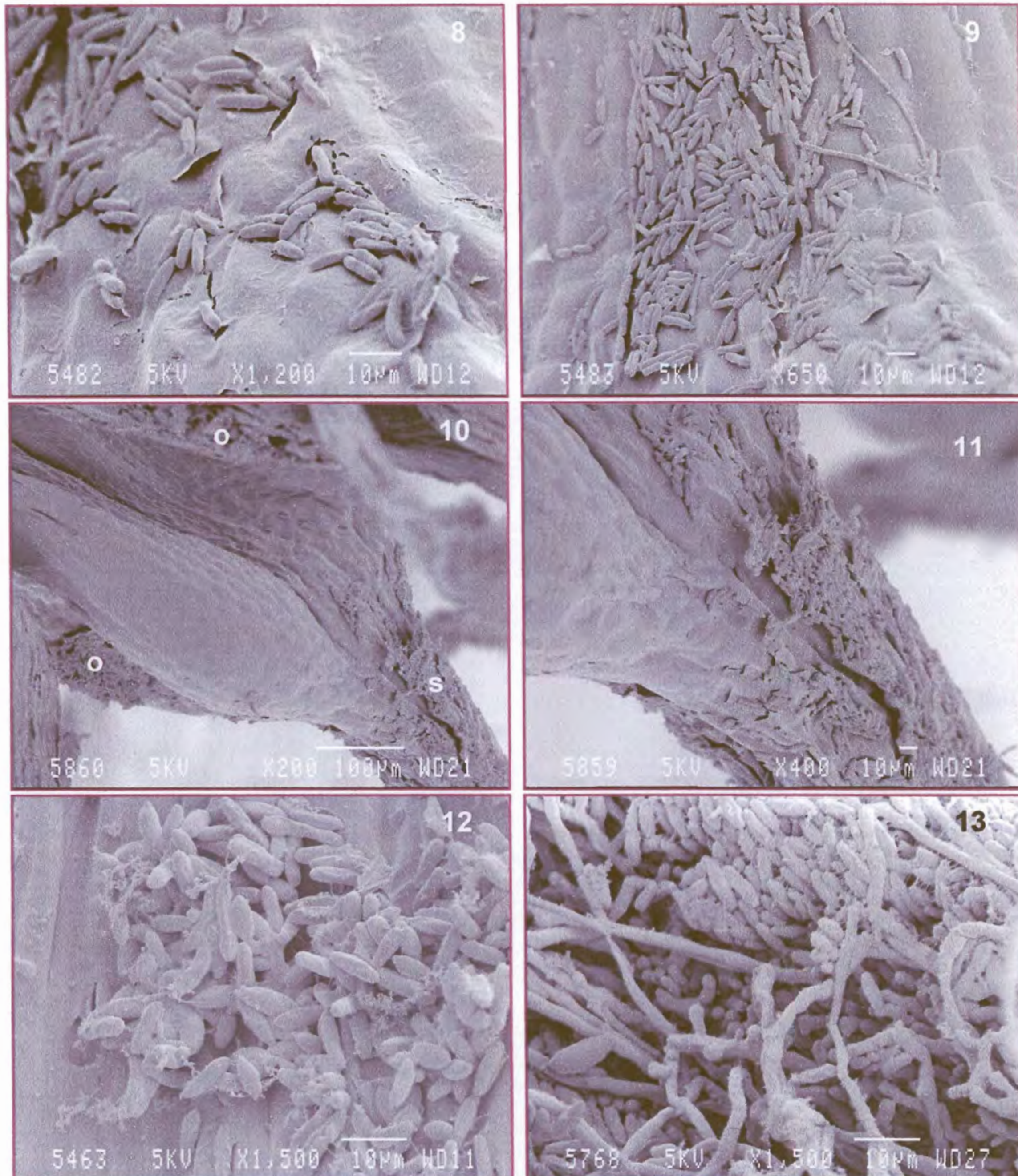


Figures 4.8-4.13 SEM micrographs of infection of *Cyperus esculentus* with *Claviceps cyperi*:

4.8 Conidia on apical part of ovary.

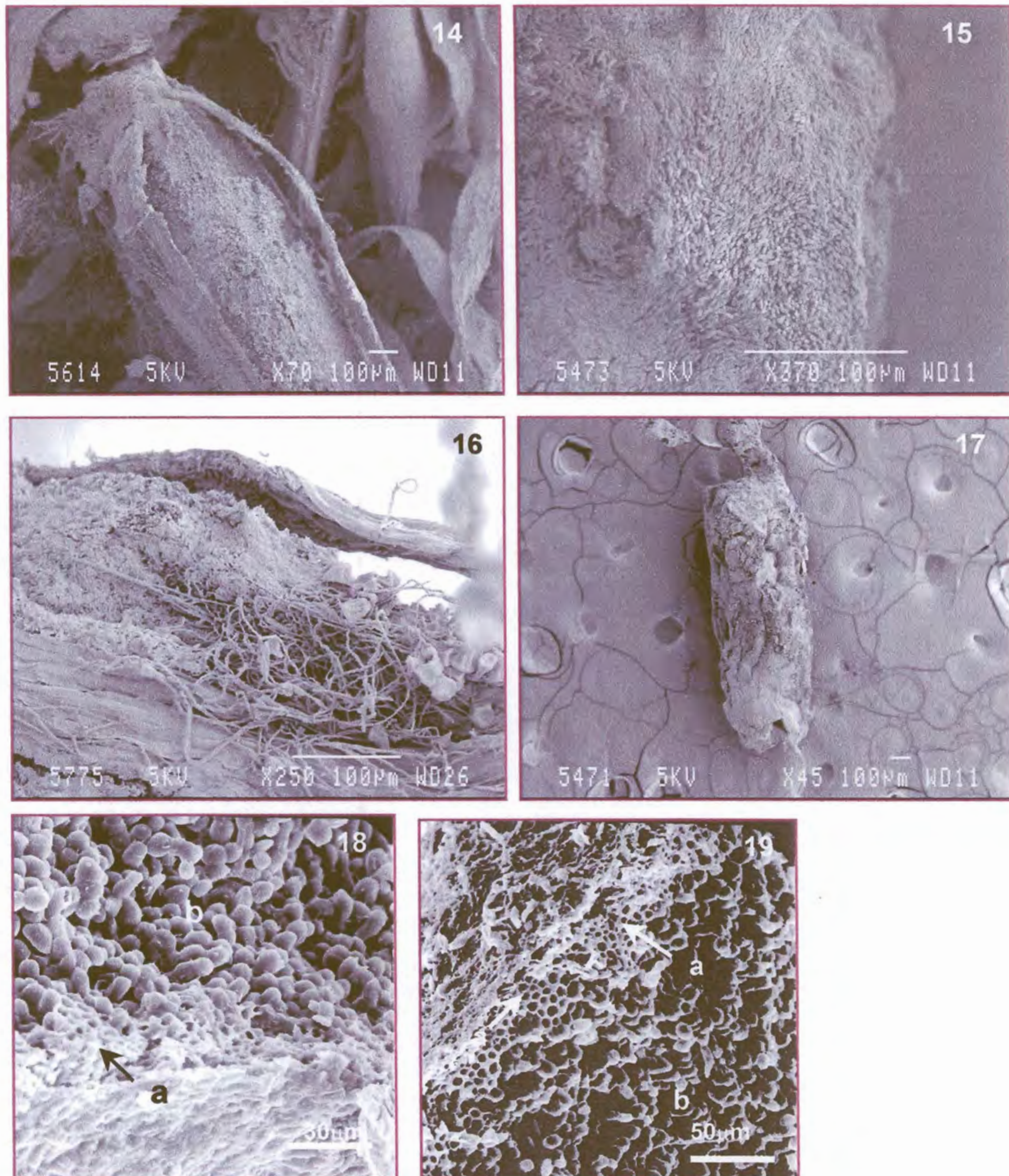
4.9 Hyphae spreading over rest of ovary.

4.10 Base of stylodium (s) and ovary (o) infected with conidia.

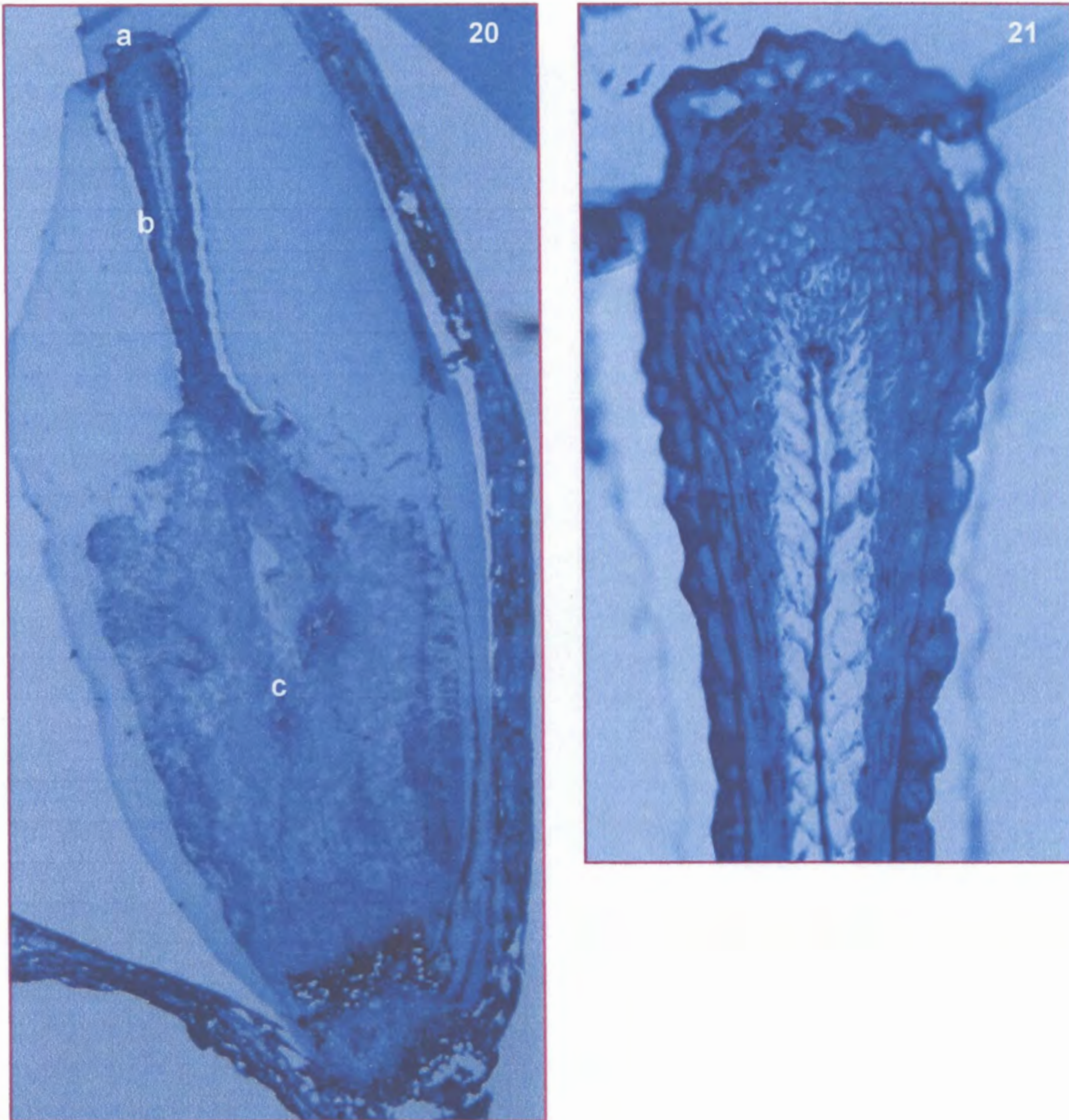
4.11 Closer view of base of stylodium and ovary neck with conidia.

4.12 *Conidial mass starting to form.*

4.13 Conidial mass with interspersed hyphae.



Figures 4.14-4.17	SEM micrographs of infection of *Cyperus esculentus* with *Claviceps cyperi:*
4.14	Ovary totally covered with conidia.
4.15	Closer view of conidial mass.
4.16	Ovary in advanced stage of infection, sclerotium beginning to develop.
4.17	Ovary completely deformed and covered with conidia with base of withered style of *Cyperus esculentus* visible at the top.
Figures 4.18-4.19	Sections through sclerotia of *Claviceps cyperi* showing difference between outer layer (a) and inner layers containing bundles of longer hyphae (b).



Figures 4.20-4.21	Light microscopy sections through florets of *Cyperus esculentus* infected with *Claviceps cyperi*:
4.20	Pistil consisting of (a) stigma, (b) stylodium and (c) ovary.
4.21	Stigma and style with no infection evident, although ovary already infected.

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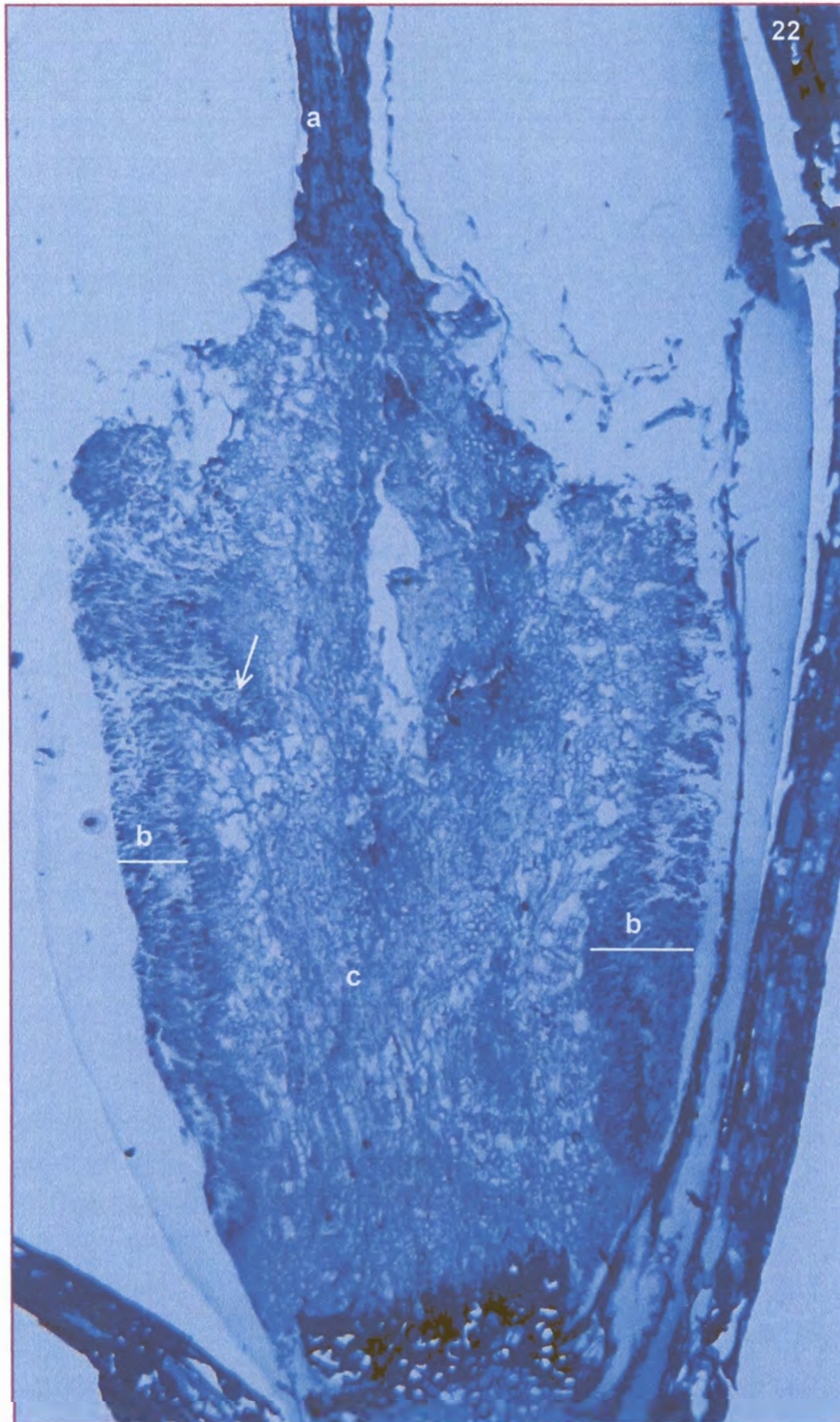

Figure 4.22 Section through pistil of *Cyperus esculentus* infected with *Claviceps cyperi*: (a) stylodium (no obvious infection), (b) conidial layer covering outside of ovary and 'labyrinthine chambers' starting to develop (arrow), and (c) hyphal cells filling ovary.

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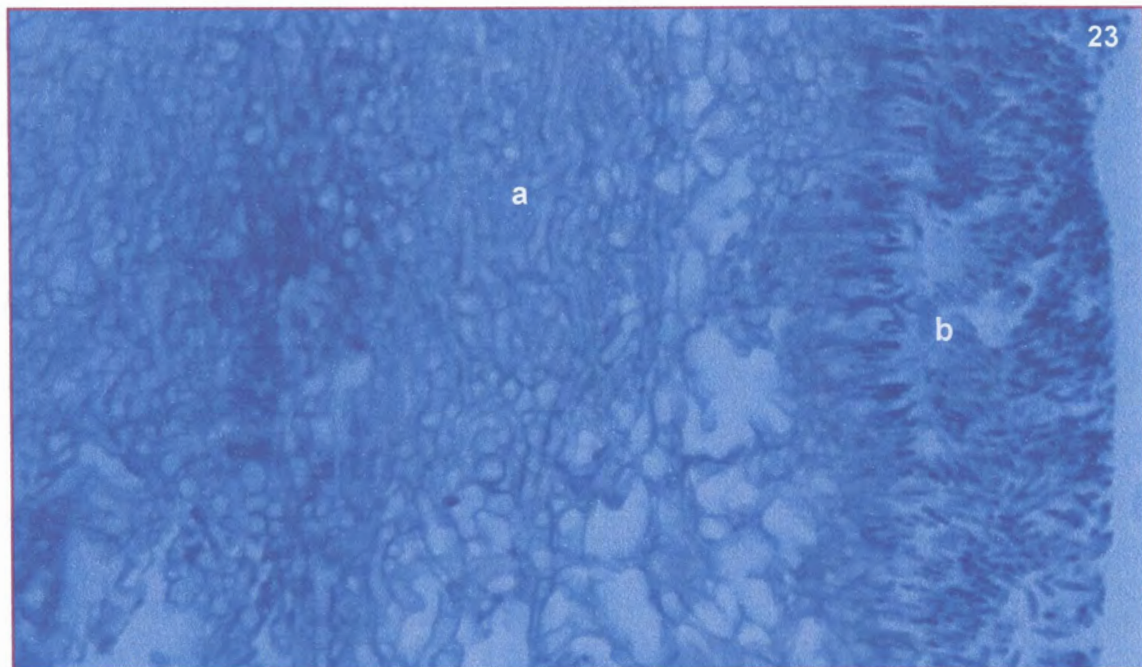
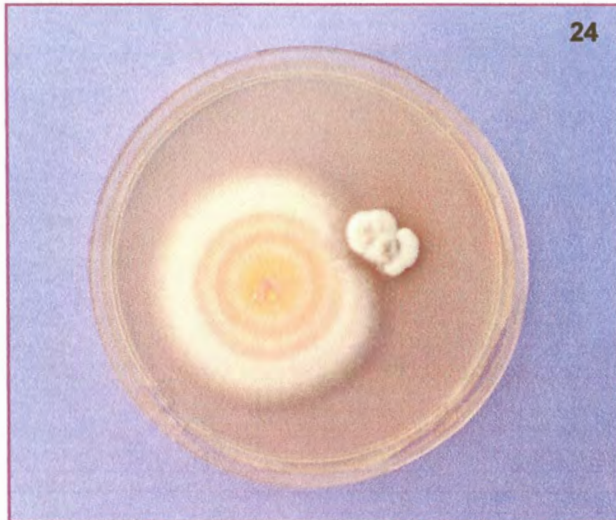
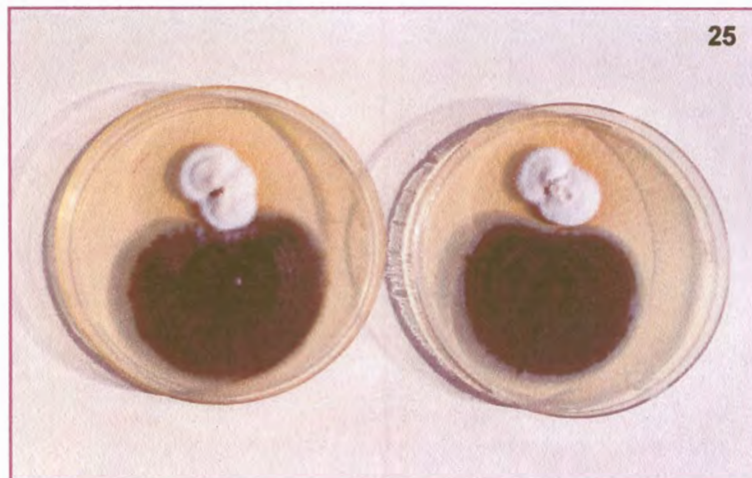


Figure 4.23 Closer view of section through ovary of *Cyperus esculentus* infected with *Claviceps cyperi*: (a) hyphal cells visible on the inside and (b) conidial layer covering ovary on the outside.





Figures 4.24-4.25 Antagonism in culture between the *Sphacelia* state of *Claviceps cyperi* and:

4.24 *Fusarium heterosporum*.

4.25 *Cladosporium cladosporioides*.

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5. ERGOT ALKALOIDS PRODUCED BY *CLAVICEPS CYPERI*

Abstract

The main ergopeptine alkaloid in sclerotia of *Claviceps cyperi* collected from ergotised nut sedge implicated in outbreaks of bovine ergotism in South Africa was identified by HPLC and tandem mass spectroscopy as α-ergocryptine. All sclerotial samples tested also yielded ergosine, whereas ergocornine and ergocrystine were detected at low concentrations in a freshly collected sample. Trace amounts of α-ergocryptine was produced by *C. cyperi* on solid medium, but all attempts at inducing an isolate of the species to synthesise ergopeptines in liquid culture have failed. α-Ergocryptine showed no antibiotic activity against *Cladosporium cladosporioides* and *Fusarium heterosporum*, two fungi commonly associated with *C. cyperi* honeydew.

5.1 INTRODUCTION

Various cases of bovine ergotism have recently been reported from the eastern Highveld Region in South Africa (Van der Linde & Naude 2000). Examination of the fodder implicated in the outbreaks showed that it was extensively contaminated with yellow nut sedge (*Cyperus esculentus* L.) ergotised by *Claviceps cyperi* Loveless, whereas chemical analysis indicated the presence of the ergot alkaloid α-ergocryptine, as well as traces of ergosine, ergocornine, ergocrystine and ergotamine (Naude *et al.* 2005).

Besides being important from a diagnostic/prognostic perspective, verification of the alkaloid or alkaloids produced by a *Claviceps* species is of value in confirming the identity of the species, defining chemoraces or chemotypes, and classifying intraspecific variability (Pažoutová *et al.* 2000), hence assisting in the elucidation of phylogenetic relationships and habitat specialisation. The potential pharmaceutical application of the particular alkaloid/s is also of considerable importance. α-Ergocryptine, for instance, is the source of 2-bromo-α-ergocryptine, a dopaminergic agent marketed world-wide under the trade name Parlodel by the firm Novartis. The drug is used, among others, for puerperal prevention or suppression of lactation, treatment of hyperprolactinaemia and prolactin-related menstrual and fertility disorders, and as an adjunct in the treatment of Parkinsonism (Snyman 2001).

2-Bromo-α-ergocryptine is presently produced semi-synthetically from wheat (*Triticum aestivum* L.) artificially infected with *Claviceps purpurea* (Fr.:Fr.) Tul.

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(http://www.dimok.de/ergot/harvest.html). However, *C. purpurea* is not a prolific producer of α-ergocryptine (Taber & Vining 1957), and *C. cyperi* may therefore be a more suitable source. This report provides data on the ergot alkaloids present in sclerotia of *C. cyperi* and describes various attempts at inducing sphacelial isolates of the species to produce α-ergocryptine in culture.

5.2 MATERIALS AND METHODS

Sclerotia of *C. cyperi* collected between 1997 and 2000 from ergotised nut sedge at localities where bovine ergotism occurred (Table 5.1) were analysed for ergot alkaloids by Meadows Cape in Paarl, Western Cape Province, according to the HPLC method of Rottinghaus *et al.* (1993), following extraction of the sclerotia as described by Scott *et al.* (1992). Sclerotia collected in 1997, 1999, 2002 and 2003 were tested in 2003 for ergopeptines as described in 5.2.3 and 5.2.4. A preliminary experiment was also done in which *C. cyperi* PREM 56618 was grown for 8 weeks at 22 °C in the dark on Mantle's alkaloid medium (Mantle 1973) solidified with 1.5 % agar, and the cultures analysed by HPLC for α-ergocryptine at the Council for Scientific and Industrial Research in Pretoria.

Following the above, *in vitro* production of alkaloids by *C. cyperi* in liquid medium was attempted according to the procedures described below.

5.2.1 Preparation of inoculum

Pure cultures of *C. cyperi* PREM 57392, subsequently deposited as PPRI 7196, were obtained by surface-disinfecting sclerotia in 1.75 % sodium hypochlorite for 5 minutes and rinsing them three times in sterile distilled water. The sclerotia were dried on sterile tissue paper and transferred to a microscope slide cleaned with 70 % ethanol. The cortex of each sclerotium was removed with a sterile surgical blade, and the inside cut into sections which were plated on potato-dextrose agar and glucose yeast extract agar. When the colonies were about 25 mm in diameter, plugs with mycelium cut from them were used to inoculate Erlenmeyer flasks with glucose yeast extract broth. The flasks were incubated stationary in the dark for 2 weeks at 22 °C and the cultures used to inoculate the media in 5.2.2.

5.2.2 Culturing

The following liquid media were prepared (concentrations are per litre):

Mantle's alkaloid medium (Mantle 1973) (MAM):

Sucrose	150 g
L-Asparagine	15 g
KH_2PO_4	25 mg
$MgSO_4.7H_2O$	25 mg
$FeSO_4.7H_2O$	33 mg
$ZnSO_4$	27 mg

(pH adjusted to 5.5 with 1 N NaOH)

Bacon's alkaloid medium (Bacon *et al.* 1979) (BAC):

Sorbitol	100 g
Glucose	40 g
Succinic acid	10 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.3 g
$FeSO_4.7H_2O$	1 mg
Yeast Extract	1 g

(pH adjusted to 5.5 with 1 N NaOH)

Molasses medium (MOL):

Molasses	150 g
Yeast extract	5 g

(pH adjusted to 5.2 with 1N NaOH)

Glucose yeast extract (Fuentes *et al.* 1964) (GYE):

Glucose	10 g
Yeast Extract	10 g

(pH adjusted to 5.2 with 1N NaOH)

Each medium was dispensed into sixty-four 250 ml capacity Erlenmeyer flasks at 100 ml per

flask, and the flasks with medium were autoclaved for 15 minutes at 121 °C. When cooled, each flask was inoculated with 1 ml of the above inoculum of *C. cyperi* and sixteen flasks of each medium were incubated stationary in the dark at 15, 22, 27 and 34 °C, respectively.

5.2.3 Extraction of alkaloids

Extractions from cultures were done weekly for 8 weeks with duplicate flasks of each medium from each incubation temperature. The content of each flask was filtered through Whatman No. 1 paper. Two aliquots of 5 ml of the filtrate were each transferred to a 15 ml screw-cap vial. Drops of 0.1 N sodium hydroxide were added to increase the pH to between 9 and 10. Nine millilitres of chloroform was added to each vial and the vials agitated on a rotary shaker for 30 minutes, after which they were centrifuged for 3 minutes at 4000 rpm. The upper aqueous layer was discarded and the remaining chloroform evaporated to dryness with nitrogen in a Reacti-Vap at 50 °C.

The mycelial mass was rinsed with distilled water, divided in two portions, and each portion was transferred to a pre-weighed 15 ml screw-cap vial. The fungal material was dried for 2—3 hours at 40 °C, the vials were weighed again, and the dry mycelial mass was calculated. Five millilitres of methanol was added to each vial, the contents sonicated for 1 hour, and the tubes then centrifuged for 3 minutes at 4000 rpm. Five millilitres of the liquid in each vial was transferred to a clean vial and concentrated to dryness with nitrogen in a Reacti-Vap at 50 °C. The entire experiment was conducted three times.

5.2.4 Alkaloid analysis

Vials with dried extracts were submitted on a weekly basis for alkaloid analysis to the Veterinary Medical Diagnostic Laboratory at the University of Missouri, Columbia, USA. Ergopeptine analysis was done by the HPLC technique of Rottinghaus *et al.* (1993). Samples were extracted with alkaline chloroform, filtered and applied to Ergosil cleanup columns (Analtech, Newark, Denmark). Following elution of pigments with acetone:chloroform (4:1), alkaloids were eluted with methanol and analysed by HPLC with fluorescence detection. The presence of ergopeptines was confirmed by treating the samples with 0.2 % acetic acid and re-analysis by HPLC for the -ine isomers. The HPLC system consisted of a Perkin Elmer LC 250 pump and ISS 200 auto sampler, with detection on a Hitachi F-1200 fluorescence

detector. A Phenomenex Luna C18 column (150 mm x 4.6 mm) was used with a mobile phase of acetonitrile:water (35:65) and a 200 mg l^{-1} solution of ammonium carbonate in distilled water.

The identity of the ergopeptine alkaloids was verified by tandem mass spectroscopy (Finnigan/MAT TSQ 70 Tandem Mass Spectrometer (MS/MS)) according to Rottinghaus *et al.* (1993) at the USDA National Veterinary Services Laboratory, Ames, Iowa. A portion of each of the chloroform extracts processed through the above Ergosil cleanup columns was applied to a direct exposure probe, the solvent allowed to evaporate, and the probe inserted into the mass spectrometer. The MS/MS was operated in the negative chemical ionisation mode with methane as reagent gas and argon as collision gas. Daughter ions were collected for the α-ergocryptine parent ion m/Z 308. Typical daughter spectra were obtained with base peak m/Z 209, in accordance with Plattner *et al.* (1983) and Rottinghaus *et al.* (1993).

5.2.5 Antimycotic activity of α-ergocryptine

Colonies of the two fungal species most commonly associated with honeydew of *C. cyperi*, viz. *Fusarium heterosporum* Nees and *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, were established centrally on potato-dextrose agar plates. When about 1 cm in diameter, four antibiotic assay discs saturated with chloroform solutions containing 2000, 3000, 4000 and 5000 ppm pure α-ergocryptine (Sigma, Johannesburg), respectively, were placed equidistantly around each colony on the agar. Plates were incubated at 18 °C in the dark and inspected regularly.

5.3 RESULTS

The main ergopeptine alkaloid detected in sclerotia of *C. cyperi* was α-ergocryptine, with concentrations in freshly collected samples ranging from 2500 to 5325 ppm (Table 5.1). When tested again in 2003, the α-ergocryptine content of the sclerotia on average was about 50 % less than in the initial analysis. All sclerotial samples tested in 2003 also contained ergosine at concentrations 3.4—24 times lower than that of α-ergocryptine. Small quantities of ergocornine and ergocristine were present in sclerotia collected and tested in 2003.

Cultures of PREM 56618 grown on solid MAM produced a dark brown diffusable pigment in

the agar (Figure 5.1). The concentration of α-ergocryptine in these cultures after 8 weeks was 7 ppb.

No growth of PREM 57392 occurred in any of the liquid media at 34 °C. Some growth was evident in MOL at 15 °C, but not in the other three media. Cultures in MAM and BAC at 22 and 27 °C started to produce a brown pigment in the medium after about 4 weeks. Best growth in terms of biomass produced at the latter temperatures (data not presented) occurred in GYE. No ergopeptine alkaloid could be detected in any of the media at any time at any temperature in any of the three runs of the experiment.

Colonies of *F. heterosporum* and *C. cladosporioides* grew unobstructedly over the antibiotic assay discs impregnated with α-ergocryptine (Figures 5.2, 5.3).

5.4 DISCUSSION

Environmental conditions play an important role in *Claviceps* fermentations (Mantle 1978; Arora *et al.* 1992; Wainwright 1992). With most strains, the metal ions Mg, Fe and Zn in trace amounts enhance alkaloid production. pH optima for growth are around 5.2—5.5, and temperature optima usually near 24 °C. Induction of alkaloid biosynthesis requires osmotic pressures between 10 and 20 bar, attained by high sugar concentrations which can be partially replaced by sodium chloride. The high osmotic pressure inhibits conidiogenesis, induces differentiation into sclerotium-like cells, and enhances the entrance of nutrients into the cells. As is common for most secondary metabolites, alkaloid synthesis follows rather than accompanies active growth. Maximum alkaloid yield therefore usually occurs only after prolonged incubation (12—50 days) (Abe 1948; Taber & Vining 1957; Mantle 1973). By adhering to these principles, alkaloid synthesis in culture has successfully been achieved with ergot species such as *C. purpurea* (Michener & Snell 1950; Taber & Vining 1957; Castagnoli & Mantle 1966), *Claviceps paspali* F. Stevens & J.G. Hall (Pacifici *et al.* 1962), *Claviceps gigantea* S.F. Fuentes, Isla, Ullstrup & Rodriques (Fuentes *et al.*, 1964), *Sphacelia sorghi* McRae (Mantle 1973), *Claviceps fusiformis* Loveless (Mantle 1978), *Claviceps africana* Freder., P.G. Mantle & W.A.J. de Milliano, *Claviceps sorghicola* Tsukib., Shiman. & Uematsu (Pažoutová 2001) and *Claviceps zizaniae* (Fyles) Pantidou (Kantorová *et al.* 2002).

Despite adhering to the above guidelines, and allowing for possible deviations in

environmental requirements, the present attempts at inducing *C. cyperi* to respond accordingly have failed, except for the trace amounts of α-ergocryptine produced on solid medium. The lack of success could probably be ascribed to an inherent inability of *C. cyperi* to synthesise ergopeptines in liquid culture or to the isolates used having lost the capacity to produce alkaloids. Von Bekesy (1940) showed that cultures of *C. purpurea* obtained from sclerotia with a high alkaloid content yielded the highest concentration of alkaloids. The two isolates of *C. cyperi* used in the present study, particularly PPRI 7196, were selected for that reason. Although alkaloid-producing cultures have an acute tendency to loose the ability to yield alkaloids upon repeated transfer (Mantle 1978), PPRI 7196, when inoculated into the various liquid media, had been subcultured only twice since being obtained in culture from PREM 57392 sclerotia. A more feasible explanation for the absence of ergopeptines in cultures of PPRI 7196 therefore seems to be that cultural conditions were not conducive to alkaloid production by this isolate of *C. cyperi*. Observations by Ramstad & Gjerstad (1955) indicated that mycelial tissue of *C. purpurea*, in the same way as conidia, is incapable of producing alkaloids, and that failure to produce alkaloids in culture is due to sclerotial tissue not developing under these conditions. Mantle & Tonolo (1968) similarly showed that improved *in vitro* alkaloid yields by *C. purpurea* is associated with a plectenchymatic growth form. Enlarged hyphal cells, probably sclerotial primordia, have been observed in cultures of *C. cyperi* on solid medium in Chapter 3, but it is not known if such cells were formed in liquid medium in the present study. Growth of the cultures certainly did not appear plectenchymatous. Regarding the above it is interesting to note that Pažoutová (2001) reported sclerotia and cultures of *C. zizaniae*, the *Claviceps* species phenotypically the closest related to *C. cyperi* (Chapter 6) and the only other ergot species producing α-ergocryptine as main alkaloid, to be void of alkaloids. In a subsequent study, however, Kantorová *et al.* (2002) found one wild strain of *C. zizaniae* to synthesise α-ergocryptine at concentrations of up to 1 mg g^{-1} in the same medium used by Pažoutová (2001), whereas a second strain did not produce any detectable alkaloids at all.

Contrary to the culture experiments, ergopeptine analysis of sclerotia of *C. cyperi* provided vital information regarding the chemistry of *C. cyperi*. More than 80 ergot alkaloids are known (Bock & Parberry, www.tacethno.com/info/claviceps Internet access 13.01.2005), but peptide ergot alkaloids (ergotamine, ergosine, ergocristine, ergocornine, ergostine, α-ergocryptine, ß-ergocryptine and derivatives) have thus far been detected only in *C. purpurea* (various ergopeptines), *C. zizaniae* (α-ergocryptine) and *C. africana* (dihydroergosine) (Mantle 1968; Flieger *et al.* 1997; Kantorová *et al.* 2002). *C. cyperi* is therefore the fourth *Claviceps* species

found capable of producing ergopeptines. This alkaloid profile corresponds with the alkaloid analysis of the fodder implicated in the outbreaks of bovine ergotism (Naude *et al.* 2005) and is typically associated with "summer syndrome" symptoms, e.g. hyperthermia, reduced food intake, lethargy, drop in milk production, loss of body mass, increased respiratory rate, open-mouthed breathing, seeking shade and wading into water (Ross *et al.* 1989). One of the fodder samples tested by Naude *et al.* (2005), however, contained ergotamine, which was not detected in sclerotia of *C. cyperi*. The presence of ergotamine in the above sample can probably be ascribed to contamination of the fodder by a *Lolium* sp. ergotised by *C. purpurea*, also occurring in the area.

Besides elucidating the alkaloid profile of *C. cyperi*, results of the present study verified the relatively narrow mesophilic temperature growth range of this ergot species (Chapter 3) and also showed that α-ergocryptine does not possess antimycotic activity against *F. heterosporum* and *C. cladosporioides*. The inhibition of growth of the latter two fungi by *C. cyperi* observed in dual culture in Chapter 3 must therefore have been due to a different metabolite produced by *C. cyperi* in the medium.

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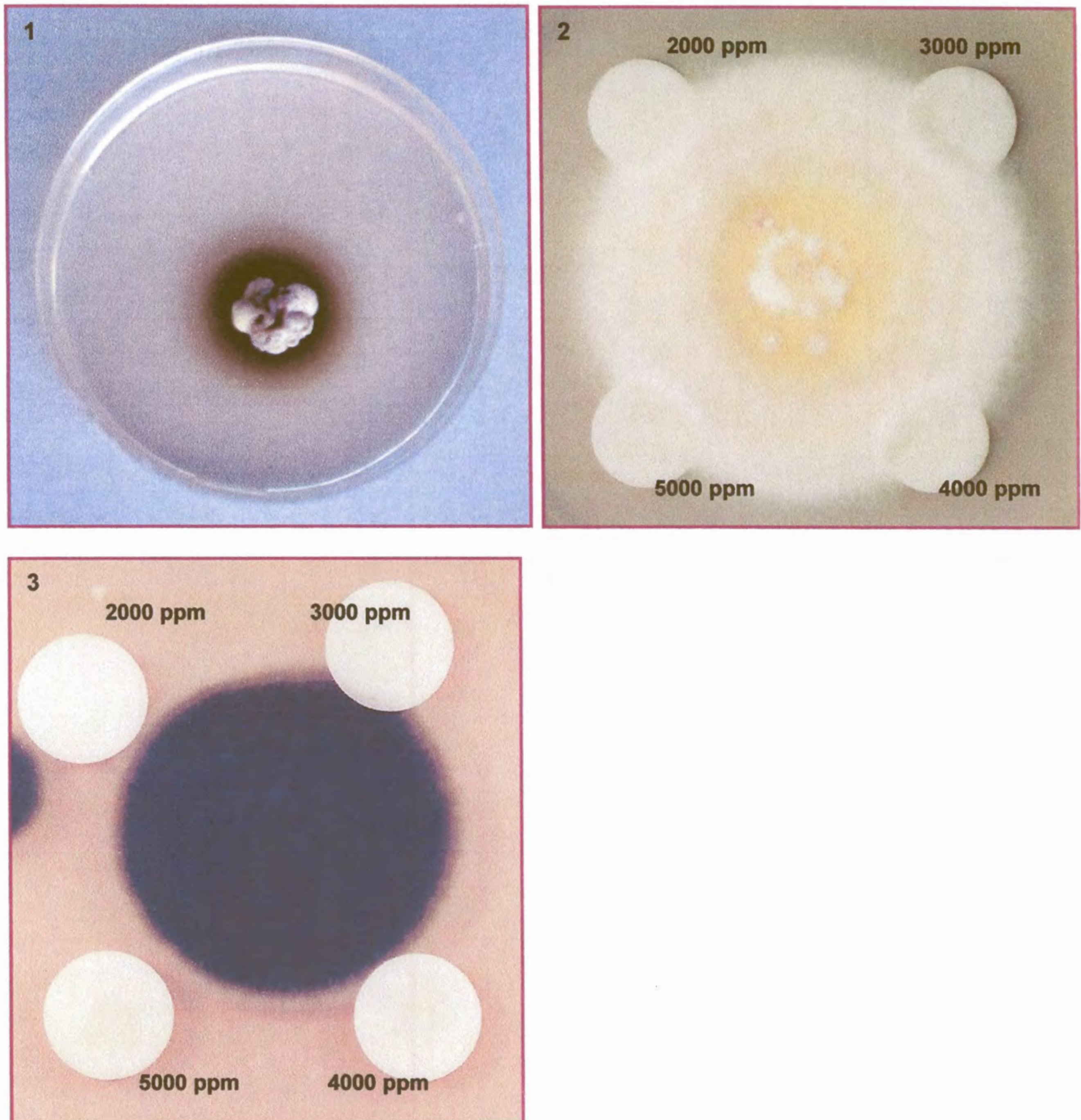

Figure 5.1 Colony of *Claviceps cyperi* growing on Mantle's alkaloid medium.
Figure 5.2 Lack of inhibition of *Fusarium heterosporum* by α-ergocryptine.
Figure 5.3 Lack of inhibition of *Cladosporium cladosporioides* by α-ergocryptine.

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Table 5.1 Ergopeptine alkaloid content of sclerotia of *Claviceps cyperi* collected between 1997 and 2003 from ergotised nut sedge implicated in bovine ergotism in South Africa.

Alkaloid	**Concentration (ppm)**					
	1997 PREM 56618	1998 PREM 56621	1999 PREM 56623	2000 PREM 57392	2002 PREM 57394	2003 PREM 58304
α-Ergocryptine[a]	3600	3925	2500	5325	ND	ND
α-Ergocryptine[b]	2491	ND	1676	ND	1902	2687
Ergosine[b]	102	ND	97	ND	147	789
Ergocornine[b]	0	ND	0	ND	0	30
Ergocristine[b]	0	ND	0	ND	0	26

[a] Determined in year of collection.

[b] Determined in 2003

ND = Not determined

6. MOLECULAR SYSTEMATICS OF *CLAVICEPS CYPERI* AND OTHER SOUTH AFRICAN *CLAVICEPS* SPECIES

Abstract

Two South African isolates of *Claviceps cyperi*, one of *C. purpurea*, and a Canadian strain of *C. grohii* (CBS 124.47) were characterised with the aid of three primers to produce multilocus fingerprints. The internal transcribed spacers 1 and 2, the 5.8S region, as well as the β-tubulin intron 3 region were sequenced. All available sequence data for several *Claviceps* species deposited in the GenBank nucleotide database were compared and optimally aligned with the South African isolates. K-means clustering and two-dimensional discriminant analysis as well as phylogenetic relationships were determined on these data sets. *C. cyperi* and *C. zizaniae* formed a distinct cluster showing little similarity with the other *Claviceps* species which clustered in two main groups. The South African isolate of *C. purpurea* and *C. grohii* (CBS 124.47) showed high similarity with the GenBank *C. purpurea* strains and clustered with *C. sulcata*, *C. fusiformis* and *C. paspali*. *C. africana* was included in the second major cluster with *C. viridis*, *C. pusilla*, *C. sorghi*, *C. gigantea*, *C. maximensis*, *C. phalaridis*, *C. sorghicola* and *C. citrina*. It was difficult to place *C. citrina* and *C. paspali* in either of the major clusters using the phylogenetic analysis. Sequence data from the β-tubulin intron 3 region revealed a similar pattern and placed the two *C. cyperi* isolates in a distinct outgroup cluster at a large genetic distance from the other *Claviceps* species.

6.1 INTRODUCTION

Claviceps species have traditionally been identified according to morphological characteristics of the teleomorph and the anamorph, as well as the hosts from which they were recorded. However, morphological features within species tend to be variable (Pažoutová *et al.* 2000b), whereas description of a new species based solely on the host is inclusive due to the polygeneric host range of the majority of ergot fungi (Pažoutová *et al.* 2000b). Attempts to circumvent the problem have led to the establishment of varieties, special forms or races, and host-specific groups (Pažoutová *et al.* 2000b; Pažoutová 2001). Other means of distinguishing between species, such as alkaloid profiles and the ability to float on water, have also been utilised to identify chemoraces and habitat specialisation (Pažoutová *et al.* 2000b, 2002).

In recent years, much emphasis has been placed on the application of molecular methods for verifying and detecting genetic variability in *Claviceps*. Most of the studies concerned *Claviceps purpurea* (Fr.:Fr.) Tul. and relied on random amplified polymorphic DNA (RAPD) analysis utilising polymerase chain reaction (PCR) techniques (Jungehülsing & Tudzynski 1997; Pažoutová & Tudzynski 1999; Pažoutová *et al.* 2000b, 2002; Duncan *et al.* 2002). Application of RAPD, random amplified microsatellite (RAM), amplified fragment length polymorphism (AFLP) and sequence analysis of the β-tubulin gene intron 3 region and EF-1-α gene intron 4 also enabled Pažoutová *et al.* (2000a) and Tooley *et al.* (2000, 2001) to separate the *Claviceps* species associated with *Sorghum* species. Although some of the above investigations included additional ergot species as outgroups, the only comprehensive phylogenetic study was by Pažoutová (2001), who compared 16 *Claviceps* species by means of PCR amplification and alignment of 5.8S rDNA and the adjacent internal transcribed spacers (ITS) 1 and 2.

One species that has thus far not been included in any molecular study is *Claviceps cyperi* Loveless, causal agent of ergot in yellow and purple nut sedge (*Cyperus esculentus* L. and *Cyperus rotundus* L.), which has recently been implicated in several cases of bovine ergotism in South Africa (Van der Linde & Naude 2000; Naude *et al.* 2005). Observations in Chapter 2 confirmed that *C. cyperi* is morphologically distinct from *Claviceps grohii* J.W. Groves and *Claviceps nigricans* Tul., the other two ergot species recorded on *Cyperaceae*. This report describes the molecular characterisation of *C. cyperi* and its separation from other *Claviceps* species available in the GenBank sequence database with the aid of multilocus PCR fingerprinting of genomic DNA and sequence analysis of the ITS1—5.8 rDNA-ITS2 and β-tubulin gene intron 3 regions.

6.2 MATERIALS AND METHODS

6.2.1 Strains examined

Details of the *Claviceps* and outgroup strains included in the study are summarised in Table 6.1.

6.2.2 Extraction and purification of DNA

The CTAB method of Ausubel *et al.* (1989) was used for the extraction and purification of genomic DNA of the four South African isolates included in this study (Table 6.1). Purified DNA was quantified with fluorometry using Hoescht (H 33258) dye and a Hoefer Dyna Quant 200 mini-fluorometer (Hoefer, San Francisco, CA). The final DNA concentration was adjusted to between 25—50 ng μl^{-1} at which all rep-PCR and RAPD fingerprinting was performed.

6.2.3 PCR fingerprinting of genomic DNA

All reactions were performed in a total volume of 10 µl in 0.2 ml microtubes. Repetitive sequence-based (rep-PCR) typing was performed with BOXA1R and ERIC2 single primers according to Versalovic *et al.* (1994). PCR buffer for rep-PCR was changed to 10 mM Tris-HCl; 50 mM KCl; 3.5 mM $MgCl_2$; 0.1 % Triton X-100; pH 9.0 (Promega, Madison, Wisconsin). A GC-rich arbitrary primer ARP-7 was used according to Mathis & McMillan (1996). PCR products were separated in 1.5 % agarose at 80 V constant in TBE 1x buffer (89 mM Tris-base; 89 mM borate; 2 mM EDTA, pH 8.0). Amplification products were stained with ethidium bromide (1 mg ml^{-1}) for 15 minutes at 25 °C and destained for 10 minutes. DNA relative molecular mass marker (Roche Diagnostics, no VI; GmbH, Mannheim) was loaded in lane one of each gel. Gel images were captured with a CCD camera and stored as TIFF-uncompressed graphic files. Gel images were processed and a similarity matrix and maximum parsimony tree created using Bionumerics ver. 3.0 (Applied-Maths, BVBA, St.-Martens-Latem). Cluster and discriminant analysis of fingerprints were performed on the similarity matrix with Ward's clustering algorithm with Euclidean distances. (Statistica ver. 6.0; Statsoft, Tulsa, Oklahoma). K-means clustering of group means was performed based on user-specified clusters as derived from the dendrogram.

This clustering method attempts to minimise the distances within each cluster and to maximise between-cluster distances. The K-means method tests the validity of user-specified clusters based on the topology of the dendrogram. In addition to K-means,

Multivariate Analysis of Variance (MANOVA) was used to test the validity of user-delineated groups (clusters). MANOVA is a statistical method which proves that the likelihood of obtaining equally good separations and discrimination when groups are chosen at random, approaches zero. In addition, MANOVA will determine which characters are responsible for the discrimination between delineated groups. Parameter L (Wilkinson's likelihood for normal distributions) predicts the likelihood that the groups as delineated by the user, were drawn from the same population. When L is low to zero, the strains or entries most likely were drawn from different populations confirming the discrimination between user-delineated groups. Parameter p is the probability that a random subdivision into different groups would produce equally good discrimination between groups. Once again a low p value will confirm the validity of user-delineated groups.

6.2.4 Sequence analysis of the ITS1/2 and 5.8S regions (rDNA operon)

This region of the rDNA operon was chosen because sequence data of several *Claviceps* species has been submitted to the GenBank database, which makes it convenient for comparative purposes when new strains are sequenced. The complete ITS 1, 5.8 S and ITS 2 regions were amplified using primers ITS 5 (5' - GGA AGT AAA AGT CGT AAC AAG G -3') and ITS 4 (5' - TCC TCC GCT TAT TGA TAT GC-3') which are complementary to conserved sequences flanking the ITS 1 and 2 spacers. A schematic presentation of the rDNA gene cluster amplified is shown in Figure 6.1. Amplification conditions were as follows: initial denaturation 95 °C for 3 minutes; annealing at 60 °C for 1 minute; extension at 72 °C for 2 minutes; 30 cycles performed at 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 2 minutes; final extension at 72 °C for 10 minutes. The reaction mixture consisted of 10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1 % Triton X-100; pH 9.0; Taq polymerase 0.133 units per 10 μl reaction mixture (Promega, Madison, Wisconsin).

PCR amplified ITS1/2 and 5.8 S regions were purified using the High Pure PCR Product Purification kit (Roche Molecular Diagnostics, Johannesburg, South Africa) or Qiagen PCR Purification kit (Southern Cross Biotechnologies, Johannesburg) according to the

manufacturer's instructions. To assess the purity and concentration of the purified product, 1.0 µl was subjected to electrophoresis on a 1 % agarose gel (Promega, Madison, Wisconsin).

The purified PCR products were sequenced directly, without any additional cloning procedures, using the ABI Prism BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (with AmpliTaqR DNA Polymerase, FS) (PE Applied Biosystems, Foster City, California). Each sequencing reaction was carried out in a 5 µl volume containing approximately 100 ng template DNA, 12.5 pmol primer and 2 µl ready reaction premix (supplied with the sequencing kit, containing the dye terminators, dNTP's, AmpliTaq DNA Polymerase, $MgCl_2$ and Tris-HCl buffer, pH 9.0).

Sequences were aligned in accordance with DNAMAN ver. 5.1 (Lynnon BioSoft, Quebec) using optimal alignment and a dynamic method according to Feng & Doolittle (1987) and Thompson *et al.* (1994). The following optimal alignment parameters were applied: gap open penalty (10.00), gap extension penalty (5.00), percentage delay divergent (40). Distance matrices were calculated using a) Observed Divergence in combination with the Kimura 2-parameter correction (Kimura 1980), and b) Maximum Likelihood (Hasegawa *et al.* 1985). A rooted phylogenetic tree was then constructed using the Neighbour-joining method of Saitou & Nei (1987). Bootstrap confidence values were determined using 500 permutations of the data set to establish confidence at branching points (Felsenstein 1985). Sequence data sets were submitted to GenBank. Accession numbers are listed in Table 6.1.

Distance matrix spreadsheets generated by DNAMAN software were analysed by Statistica ver. 6.0 (Statsoft, Tulsa, Oklahoma). Dendrograms depicting grouping of species were drafted using Ward's clustering algorithm with Euclidean distances. The K-means clustering procedure was used to place species in well-defined clusters or groups showing distances between clusters and the distance from the cluster centre of each strain within each cluster. Between-cluster distances were maximised and within-cluster variance minimised.

Non-metric multidimensional scaling was applied to detect meaningful hidden dimensions that reveal observed similarities and dissimilarities (distances) between strains and species. A two-dimensional scatterplot is constructed to show clusters of data points and other particular patterns of relatedness between strains and species in a two-dimensional plane. Principal component analysis is performed (standard Guttman-Lingoes) as a first step, followed by measures of goodness-of-fit or the raw stress value, which measure how well a particular configuration reproduces the observed distance matrix. Stress values are presented as D-hat, D-star, alienation and a final stress value. The lower the stress value, the better the fit of the reproduced distance matrix to the observed distance matrix. The Shepard diagram is used to test the goodness-of-fit of the data points (D-hat values) on the step-function of the diagram. The closer the fit of the data points to the step function, the better the reproduction of the distances in the input data as applied to the different dimensions. Deviations from this step-function indicate lack of fit. In order to arrive at an interpretable solution, six to nine dimensions were computed followed by comparison of dimension one with dimensions two to five. The final configuration placing the strains in distinct clusters and showing the optimal separation between strains was selected.

6.2.5 Amplification and sequencing of the β-tubulin gene intron 3 region

The β-tubulin (tub2) intron 3 region was amplified using primers complementary to conserved exonic sequences (Annis & Panaccione 1998). Forward primer BT5: 5'-GCT CTA GAC TGC TTT CTG GCA GAC C-3'; reverse primer BT3: 5'-CGT CTA GAK GTR CCC ATA CCG GCA-3'; redundancies K=G/T; R=A/G. A schematic presentation of the intron 3 region amplified is shown in Figure 6.2. Amplification conditions were as follows: initial denaturation 95 °C for 1 minute; 30 cycles of 94 °C for 15 seconds; 55 °C for 15 seconds and extension at 72 °C for 15 seconds; final extension at 72 °C for 6 minutes. PCR products were gel-purified and sequenced with an automated sequencer as described for the ITS1/2 5.8S region. Sequences were aligned and data presented as described for the ITS1/2 region spacers.

Distance matrix spreadsheets generated by DNAMAN software were analysed by Statistica ver. 6.0 (Statsoft, Tulsa, Oklahoma). Dendrograms depicting grouping of

species were drafted using Ward's clustering algorithm with Euclidean distances. The K-means clustering procedure was used to place species in well-defined clusters or groups showing distances between clusters and the distance from the cluster centre of each strain within each cluster. Between-cluster distances were maximised and within-cluster variance minimised. Multidimensional scaling was performed to produce two-dimensional scatterplots as described for the analysis of the ITS 1,2 spacers.

6.3 RESULTS

6.3.1 Multilocus PCR fingerprinting of genomic DNA

Fingerprinting with all three primers clearly distinguished between *C. cyperi*, *C. grohii* (CBS 124.47) and *C. purpurea*. The dendrogram (Figure 6.3) and similarity matrix (Table 6.2) indicated a low level of similarity between these *Claviceps* species. Using BOXA1R (Figure 6.4), ARP7 (Figure 6.5) and ERIC2 (Figure 6.6) primers, *C. grohii* (CBS 124.47) clustered closer to C. *pupurea* (53 %) and showed 44 % similarity with *Tilletia indica* Mitra, while the two *C. cyperi* isolates clustered separately from both *C. grohii* (CBS 124.47) and *C. purpurea*, showing 96 % similarity between them (Table 6.2). The maximum parsimony tree (Figure 6.7) shows the branching distances between the different strains and in particular the longer distance between *C. cyperi* and the rest of the tree. All strains showed unique patterns for each primer except the two *C. cyperi* strains, which proved the usefulness of repetitive sequence-based PCR and GC-rich arbitrary primers for grouping and distinguishing between *Claviceps* species up to strain level (Figures 6.4 — 6.6).

MANOVA discriminant analysis confirmed the validity of the dendrogram clusters (Figure 6.3, Table 6.2). The likelihood that the strains of *Claviceps* species were drawn from the same population, indicating no discrimination between the strains, was very low as indicated by parameter L. The probability that a random subdivision in groups will yield the same degree of discrimination as the user-selected groups, was negligible as indicated by $P<0.001$ (Table 6.2).

6.3.2 Sequence analysis of ITS1/2 and 5.8 S regions

Strains used to construct a phylogram are listed in Table 6.3. Amplification of the complete ITS 1 and 2 spacers with the 5.8S gene produced only one PCR product for the isolates of *C. cyperi* (01/CC; 02/CC) of size 656 bp and 654 bp respectively. *C. grohii* (CBS 124.47) showed a product of 585 bp size, whereas a product of size 584 bp was amplified from *C. purpurea* (03/CP). Both *C. cyperi* strains possessed an extended ITS 1 spacer in comparison with *C. purpurea* 03/CP and *C. grohii* (CBS 124.47). No polymorphisms or other non-specific fragments were amplified. When *C. cyperi* (01/CC; 02/CC) and *C. purpurea* (03/CP) strains were compared to other *Claviceps* species deposited in the GenBank sequence database, a dendrogram (Figure 6.8) placed the *Claviceps* species in four major clusters. The related teleomorphic species of *Atkinsonella*, *Epichloë* and *Echinodothis* were included in a cluster (no. 4), together with *Claviceps citrina* Pažoutová, Fuík., Leyva-Mir & Flieger, even though the distance from the cluster centre of the latter species was larger than the distances of the other genera in this cluster (Table 6.3). *Claviceps zizaniae* (Fyles) Pantidou shows 93 % similarity in the similarity matrix with the two *C. cyperi* isolates and was consequently included in a cluster (no. 3), together with the *C. cyperi* isolates which were clearly distanced from all the other clusters according to the table of Euclidean distances (Table 6.3). *C. grohii* (CBS 124.47) and the four *C. purpurea* strains were included in a second major cluster. *Claviceps paspali* F. Stevens & J.G. Hall did not fit well in any of the four clusters (Figure 6.8, Table 6.3), because of a much larger distance from the centre of cluster 2. *Claviceps fusiformis* Loveless, *Claviceps africana* Freder., P.G. Mantle & W.A.J. de Milliano, *Claviceps viridis* Padwick & Azmatullah, *Claviceps gigantea* S.F. Fuentes, Isla, Ullstrup & Rodriquez, *Claviceps sorghi* B.G.P. Kulk., Seshadri & Hegde, *Claviceps sorghicola* Tsukib., Shiman. & T. Uematsu, *Claviceps maximensis* T. Theis, *Claviceps pusilla* Ces. and *Claviceps phalaridis* J. Walker formed the largest major cluster. In the dendrogram (Figure 6.8), *C. gigantea, C. sorghi and C. fusiformis* formed a smaller sub-cluster within this major cluster.

The two-dimensional plot (Figure 6.9) also revealed four major clusters with multidimensional scaling. Six dimensions were computed showing low stress values indicating goodness-of-fit of data points to the step function of the Shepard diagram

(Table 6.3). The first major cluster included the same nine *Claviceps* species as revealed by the dendrogram. All species in this cluster were grouped in a separate cluster (Figure 6.9). The second major cluster contained *C. purpurea*, *C. grohii* (CBS 124.47) and *C. paspali*, with *C. paspali* and one *C. purpurea* strain slightly further distanced from the cluster centre (Table 6.3). The teleomorphic species formed a less closely spaced cluster in the two-dimensional plot while *C. citrina* did not fit well in any cluster (Figure 6.9) as confirmed by the distance from the cluster centre (Table 6.3). The *C. cyperi* isolates and *C. zizaniae* formed a separate cluster well distanced from the other clusters with *C. zizaniae* further distanced from this cluster centre. The groupings generated by multidimensional scaling were similar to the clustering pattern of the dendrogram and supported the K-means partitioning of clusters.

A similar clustering pattern of four major clusters is depicted in the phylogenetic tree (Figure 6.10) which places the species in the same major clusters, except *C. paspali* which forms an outlier and occupied an intermediate position distanced from the major clusters. This positioning of *C. paspali* was more pronounced in the phylogenetic tree than in the dendrogram (Figure 6.8), the K-means table of distances (Table 6.3) and the two-dimensional plot (Figure 6.9). *C. cyperi* together with *C. zizaniae* formed a distinct separate cluster at a large distance from the other major clusters. It was apparent from the phylogenetic tree (Figure 6.10) that the related teleomorphic species, as well as *C. citrina*, were included in a single cluster, clearly separated from the other clusters as well as from *C. paspali*. All branches linking major clusters were well supported by bootstrapping.

6.3.3 Sequence analysis of the β-tubulin gene intron 3 region

Using the described amplification conditions, the β-tubulin (tub2) gene of similar size was amplified from all isolates. The isolates of *C. cyperi* 01/CC and 02/CC amplified genes of 477 bp and 473 bp size, respectively. *C. grohii* (CBS 124.47) amplified a tub2 gene of 464 bp and *C. purpurea* 03/CP a tub2 gene of 475 bp. A dendrogram presentation indicates five distinct clusters, with the two related species of *Echinodothis* and *Epichloë*, not fitting well in any of the major clusters (Figure 6.11). *C. grohii* (CBS 124.47), *C. purpurea* 03/CP and other GenBank *C. purpurea* strains formed one cluster

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(no. 4). The three *C. africana* strains formed a separate cluster (no. 3), and the *C. sorghicola* strains grouped in cluster no. 5 together with *C. fusiformis* which clustered at a larger distance from this cluster centre (Table 6.4). The two *C. paspali* strains were placed in cluster no. 2. *Epichloë* did not fit well in any cluster but linked at a larger distance with cluster no. 2. The *C. cyperi* isolates were placed in cluster no. 7, with *Echinodothis* linking at a much larger distance with this cluster (Figure 6.11). Similar to the ITS1/2 spacers sequence data, the intron 3 region also placed the *C. cyperi* isolates at the largest distance from the other species clusters as indicated by Euclidean distances (Table 6.4).

The two-dimensional plot (Figure 6.12) confirmed the clustering pattern of the dendrogram and phylogram showing the same clusters clearly distanced (Table 6.4). The *C. cyperi* isolates formed a distinct separate cluster at a large distance from the other clusters (Table 6.4). The *Echinodothis* and *Epichloë* species formed outliers and remained unclustered (Figure 6.12).

The phylogenetic tree (Figure 6.13) corresponded with the cluster pattern of the dendrogram (Figure 6.11), although the *Epichloë* and *Echinodothis* species did not fit in any cluster. As expected, the *C. cyperi* strains formed a distinct outgroup cluster (94 % sequence similarity between strains) while *C. purpurea* 03/CP and *C. grohii* (CBS 124.47) (96 % sequence similarity), clustered with other *C. purpurea* strains. The different *Claviceps* species grouped in separate clusters whereas *C. fusiformis* was only linked at a distance to the larger cluster containing *C. africana* and *C. sorghicola*. All branches linking major clusters were well supported by bootstrapping.

6.4 DISCUSSION

In this study, multilocus fingerprinting clearly differentiated between strains of *C. purpurea*, *C. grohii* (CBS 124.47) and *C. cyperi*, with the two *C. cyperi* isolates showing identical band patterns. However, due to the limited number of isolates available, variation within the species could not be established. Previous studies revealed considerable intraspecific genetic diversity within various species of *Claviceps* (Jungehülsing & Tudzynski 1997; Pažoutová & Tudzynski 1999; Pažoutová *et al.* 2000a,b, 2002; Tooley *et al.* 2000). The

above investigations indicated that multilocus fingerprinting could differentiate up to strain level, rendering it appropriate for the study of genetic characteristics of populations of ergot fungi. The lower similarity between *C. purpurea* and *C. grohii* (CBS 124.47) shown by multilocus fingerprinting in the present study, contrasted with sequence analysis of the ITS1/2 spacers and β-tubulin gene, where higher sequence similarity was established between these species. This observation is in agreement with the study by Tooley *et al.* (2000) where multilocus genotyping revealed greater genetic variation at the intraspecific level than sequence data of different genes. The random method of fragment amplification by single primers comlementary to repeated sequence motifs that are spread over the whole of the genome, should explain this intraspecific diversity.

The phylogenetic relationships indicated by the ITS1/2 spacers corresponded with those described by Pažoutová (2001), in which two distinct clades were observed, one comprising *C. citrina*, *C. phalaridis*, *C. sorghicola*, *C. sorghi*, *C. gigantea*, *C. africana*, *C. viridis* and *C. pusilla*, and the other *C. paspali*, *C. zizaniae*, *C. grohii*, *C. sulcata*, *C. fusiformis* and *C. purpurea*. In the present study, however, *C. zizaniae* showed a higher sequence similarity to the two *C. cyperi* isolates, with which it formed a distinct separate cluster based on ITS spacers sequence data. It is significant that the extended ITS1 spacer present in the *C. cyperi* isolates was also present in *C. zizaniae*. The evolutionary significance of this sequence insertion in ITS spacer 1 indicates either a common ancestor (monophyletic origin) or a paraphyletic origin of *C. zizaniae* and *C. cyperi*, though a process of convergent evolution or a speciation event could also have been involved.

Physiologically, *C. cyperi* and *C. zizaniae* share a common alkaloid profile (Kantovora *et al.* 2002; Chapter 5). Both also have hosts that prefer a moist habitat, viz. yellow nut sedge (*Cyperus esculentus* L.) and wild rice (*Zizania palustris* L. and *Zizania aquatica* L.), respectively (Loveless 1967; Pantidou 1959). Morphologically, however, the two species are quite distinct, with *C. zizaniae* producing considerably larger sclerotia than *C. cyperi*, and a differently coloured capitulum without a collar-like appendage surrounding the base (Pantidou 1959; Loveless 1967; Chapter 2). The above differences and similarities support the view of Pažoutova *et al.* (2000b, 2002), Pažoutová (2001) and Duncan *et al.* (2002) that the phylogenetic positions of *Claviceps* species tend to correspond with their alkaloid profiles and ecological specialisation rather than their morphological features and host

specificity. Further evidence regarding the unrelatedness of morphology and phylogeny in *Claviceps* can be derived from the prominent ranunculoid papilla present in *C. cyperi*, a feature it shares with *Claviceps ranunculoides* A. Möller recorded from *Setaria* sp. in Brazil (Möller 1901), that distinguishes them from all other known *Claviceps* species. No type material or GenBank strain of *C. ranunculoides* was available for inclusion in the present study. However, the species was included, together with *C. africana*, *C. fusiformis*, *C. paspali* and *C. purpurea*, in a phylogenetic analysis of the 26S large subunit rDNA of graminicolous *Clavicipitaceae* by Sullivan *et al.* (2001). According to the above analysis, *C. ranunculoides* clustered closest to *C. africana* followed by *C. fusiformis*, with *C. paspali* and *C. purpurea* forming a separate cluster. This pattern implies that *C. ranunculoides* probably would have clustered with *C. africana*, *C. fusiformis*, *C. gigantea*, *C. maximensis*, *C. phalaridis*, *C. pusilla*, *C. sorghi*, *C. sorgicola* and *C. viridis*, at a far distance from *C. cyperi*, had it been included in the present study.

Besides confirming *C. cyperi* to be a distinct species, the present investigation also provided evidence regarding the phylogeny of two "dubious" *Claviceps* species, viz. *C. citrina* and *C. paspali*. As indicated above, Pažoutová (2001) placed *C. citrina* in a cluster which included *C. africana* and *C. sorghicola*, whereas *C. paspali* grouped in a separate cluster with *C. fusiformis*, *C. grohii*, *C. purpurea*, *C. sulcata* and *C. zizaniae*. However, in a study using PCR amplification, Duncan *et al.* (2002) found *C. citrina* to cluster separately from *C. africana* and *C. sorghicola*. This grouping corresponds with the present results according to which *C. citrina* was not properly associated with any cluster, but only grouped with *Echinodothis tuberiformis* (Berk. & Ravenel) G.F. Atk., *Epichloë typhina* (Pers.) Tul. & C. Tul. and *Atkinsonella hypoxylon* (Peck) Diehl at a much larger distance. The present study also showed *C. paspali* to cluster separately from *C. fusiformis* and *C. purpurea*, which is in agreement with the sequence analysis of the β-tubulin intron 3 and EF-1 α-gene intron 4 regions reported for *Claviceps* species on sorghum by Tooley *et al.* (2001). However, in the latter study *C. fusiformis* grouped with *C. sorghicola* and *C. africana* with a bootstrap value of 82 %, possibly because resequencing of the original RAPD and ITS1 sequences reported by Pažoutova *et al.* (1998) for the strain of *C. fusiformis* referred to by Tooley *et al.* (2001) indicated that it is actually *C. purpurea* (S. Pažoutova, pers. com.). All publications based on the incorrect sequence should therefore be reconsidered.

In conclusion, although this study elucidated the phylogeny of the nut sedge ergot pathogen, extensive genetic diversity studies on South African isolates of *C. cyperi* still need to be done to establish the genetic profile of local populations of the species. The most perplexing observation was the outgroup cluster of *C. cyperi* that linked at a large distance from the other *Claviceps* species. The presence of the extended ITS1 spacer not present in any other *Claviceps* species except *C. zizaniae*, may explain this outgroup placement insufficiently. The true natural phylogenetic relationship of *C. cyperi* and *C. zizaniae* will only be resolved after a comprehensive study of many isolates from various geographic areas.

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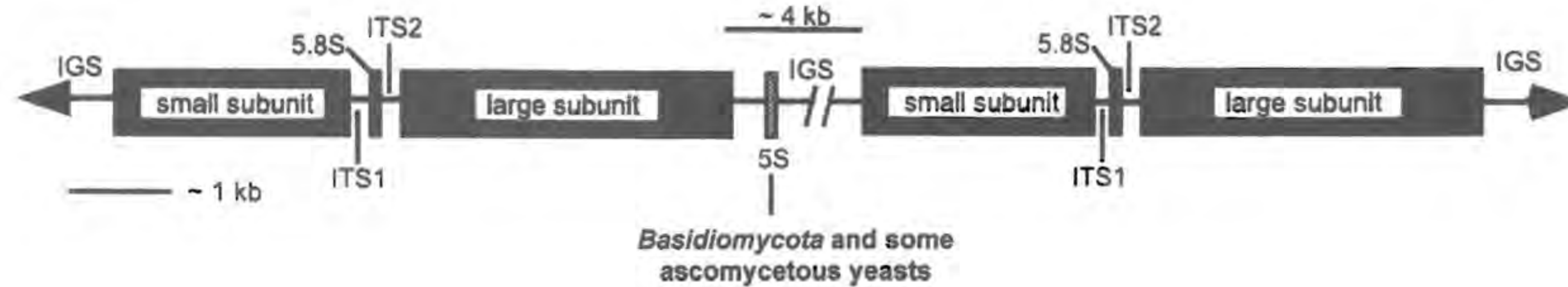

Figure 6.1 Schematic presentation of rDNA cluster of tandemly repeated ribosomal genes. Large subunit = 28 S; small subunit = 18 S; IGS = intergenic spacer; ITS = internal transcribed spacers 1 & 2; 5.8 subunit.

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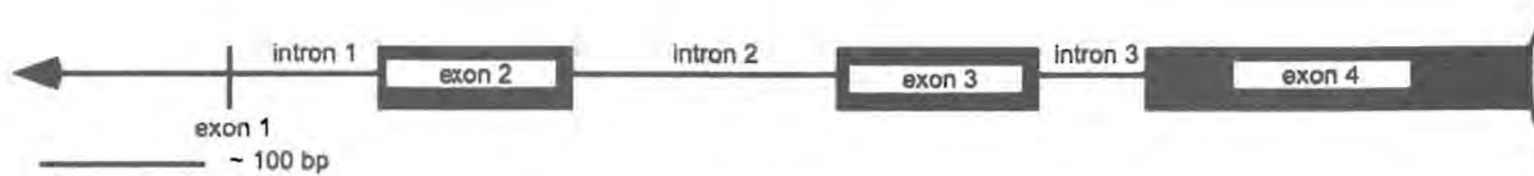

Figure 6.2 Example of an evolutionary conserved intron-rich protein-coding gene with exons 2,3,4 = conserved protein-coding sequences; introns 1-3 = variable sequences.

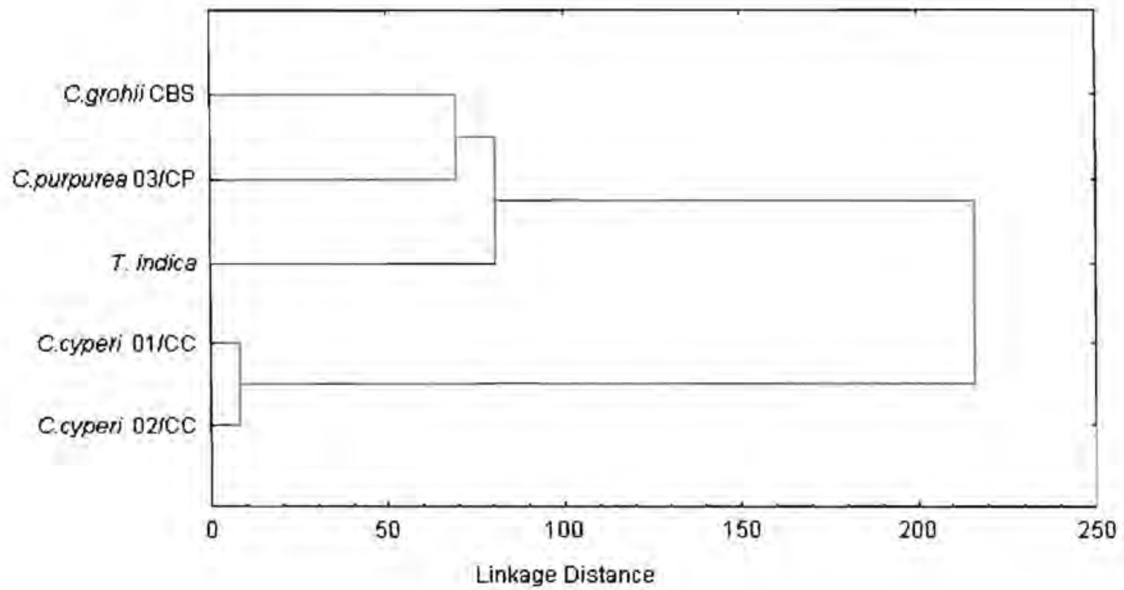

Figure 6.3 Dendrogram showing genetic differences between three *Claviceps* species and *Tilletia indica* based on multilocus fingerprinting data.

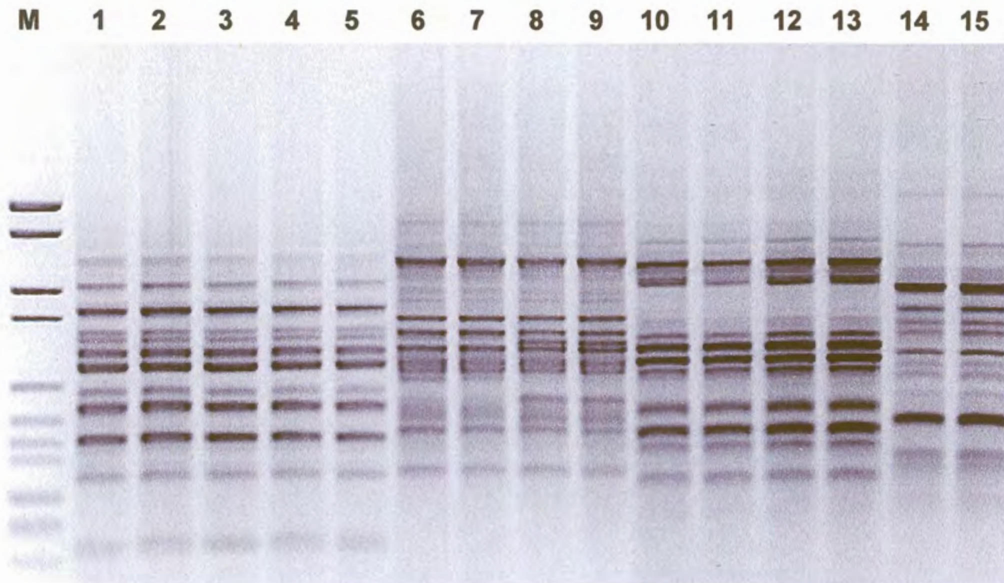

Figure 6.4 Electrophoretic band patterns using BOXA1R primer.

Lanes:	M	Molecular mass marker
	1-5	*Claviceps purpurea* 03/CP
	6-7	*C. cyperi* 02/CC
	8-9	*C. cyperi* 01/CC
	10-13	*C. grohii* CBS 124.47
	14-15	*Tilletia indica* 1325

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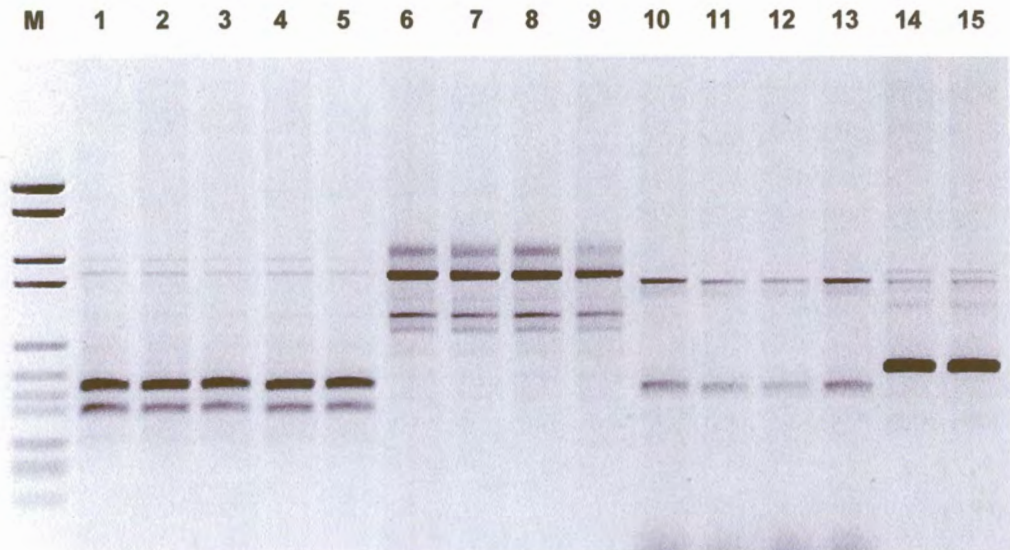

Figure 6.5 Electrophoretic band patterns using ARP-7 primer.

Lanes:
M Molecular mass marker
1-5 *Claviceps purpurea* 03/CP
6-7 *C. cyperi* 02/CC
8-9 *C. cyperi* 01/CC
10-13 *C. grohii* CBS 124.47
14-15 *Tilletia indica* 1325

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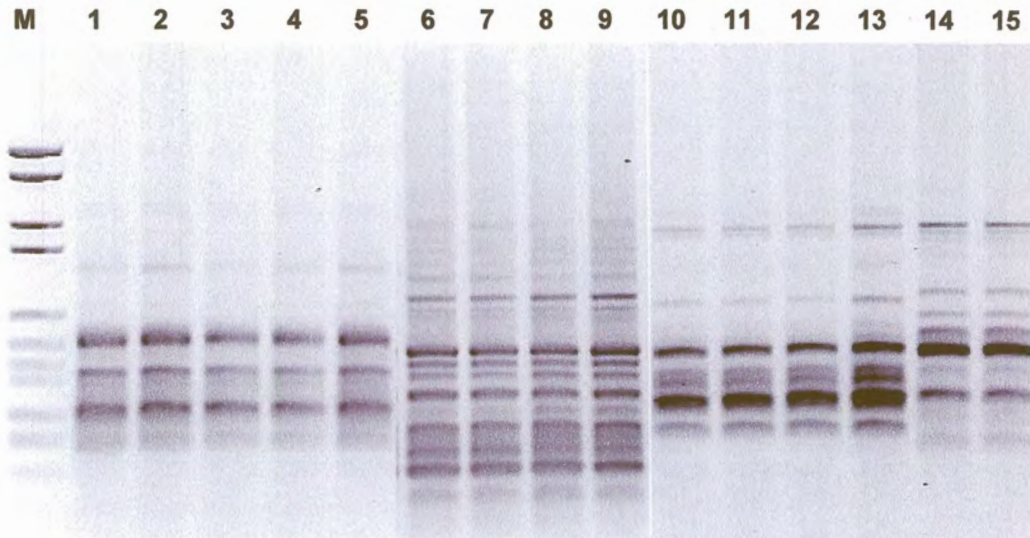

Figure 6.6 Electrophoretic band patterns using ERIC2 primer.

Lanes:

M	Molecular mass marker
1-5	*Clavicpes purpurea* 03/CP
6-7	*C. cyperi* 02/CC
8-9	*C. cyperi* 01/CC
10-13	*C. grohii* CBS 124.47
14-15	*Tilletia indica* 1325

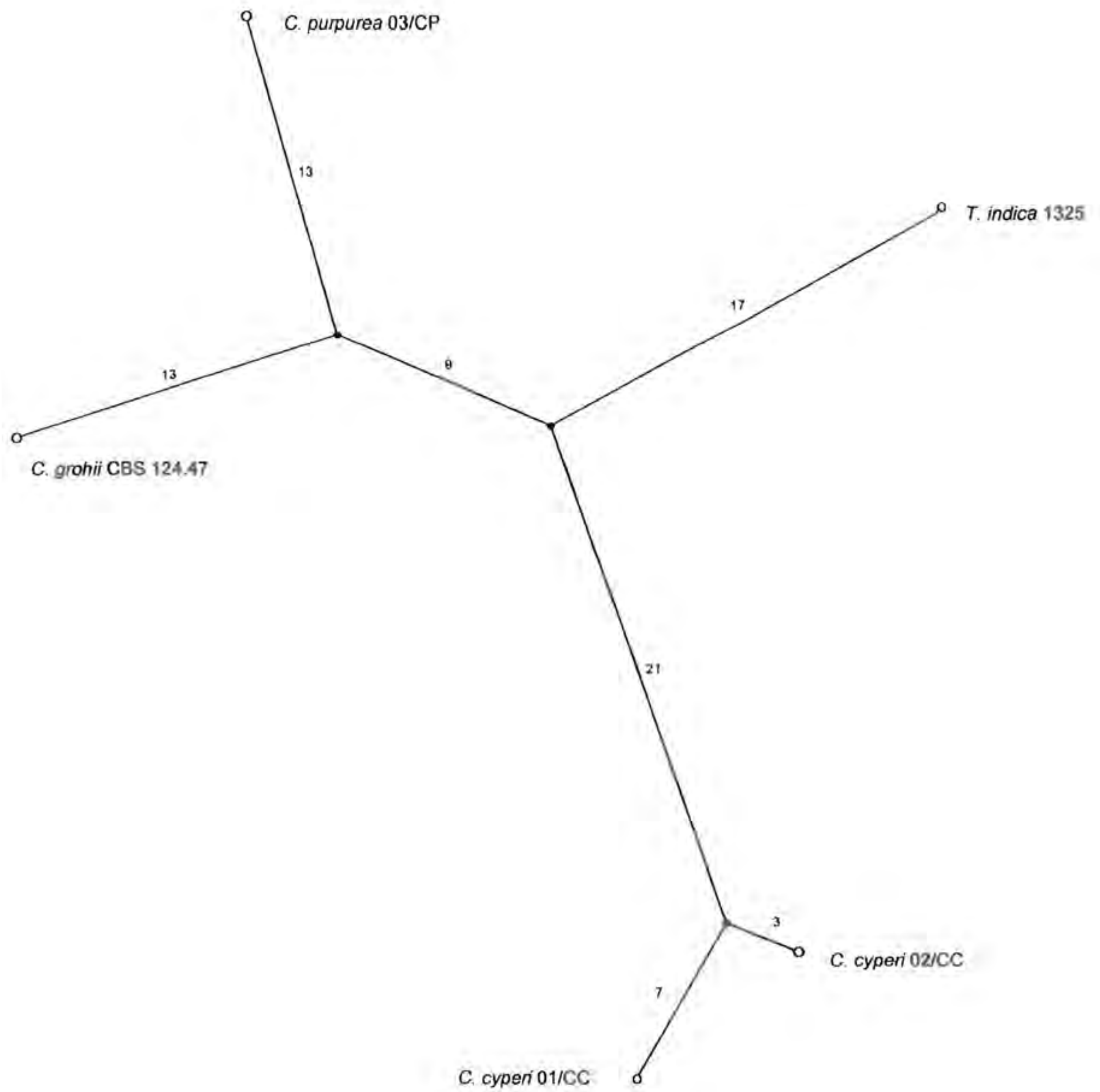

Figure 6.7 Maximum parsimony tree based on multilocus fingerprinting profiles of three *Claviceps* species and *Tilletia indica*.

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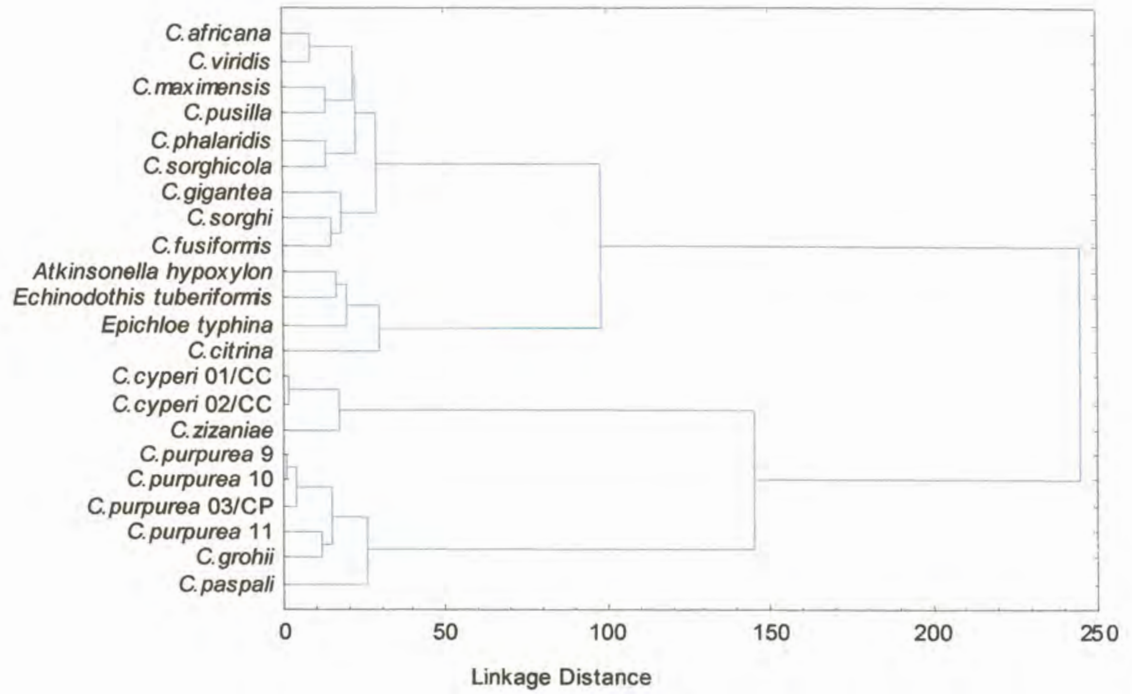
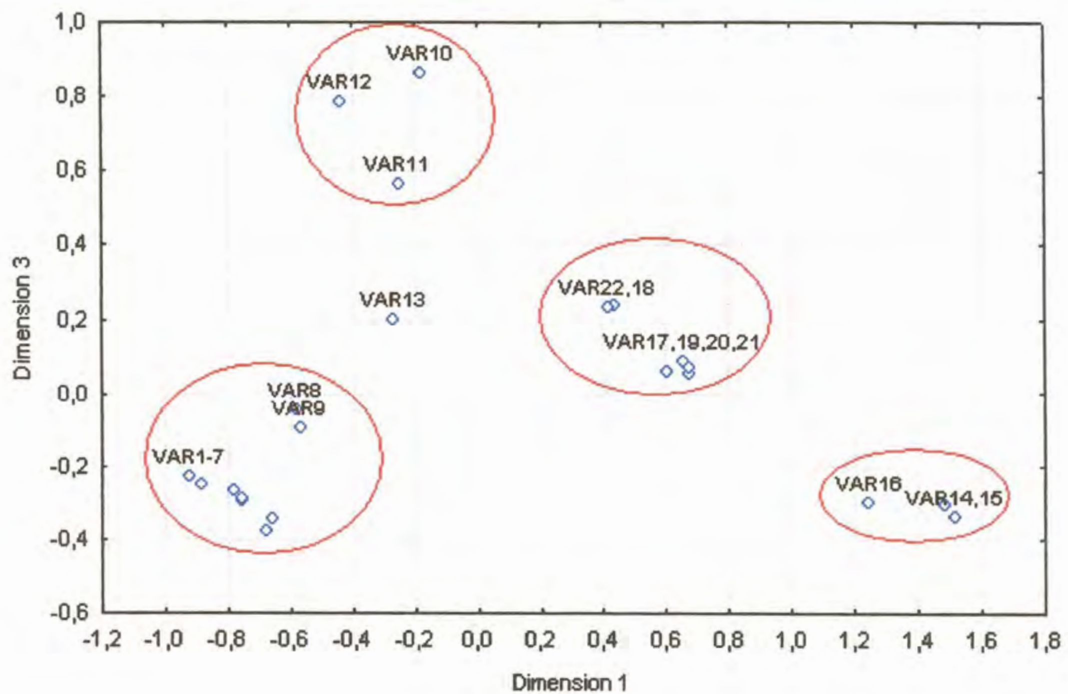


Figure 6.8 Dendrogram showing genetic distances between different *Claviceps* species and related teleomorph species based on ITS1/2 spacer sequences.



Strain code:

VAR 1	*C. africana*	NE1; Cafr
VAR 2	*C. viridis*	CBS 125.63
VAR 3	*C. gigantea*	
VAR 4	*C. sorghi*	
VAR 5	*C. fusiformis*	SD 85
VAR 6	*C. maximensis*	PM
VAR 7	*C. pusilla*	BRIP 26571
VAR 8	*C. phalaridis*	DAR 69619
VAR 9	*C. sorghicola*	
VAR 10	*Atkinsoniella hypoxylon*	CBS 125.63
VAR 11	*Echinodothis tuberiformis*	
VAR 12	*Epichloë typhina*	
VAR 13	*C. citrina*	
VAR 14	*C. cyperi*	01/CC
VAR 15	*C. cyperi*	02/CC
VAR 16	*C. zizaniae*	CCM 8231
VAR 17	*C. purpurea* 9	134 AU
VAR 18	*C. purpurea* 11	
VAR 19	*C. purpurea*	03/CP
VAR 20	*C. purpurea* 10	
VAR 21	*C. grohii*	CBS 124.47
VAR 22	*C. paspali*	MAL-1

Figure 6.9 Two-dimensional scatterplot showing genetic differences between *Claviceps* species and related teleomorphic species based on ITS1/2 spacer sequence data.

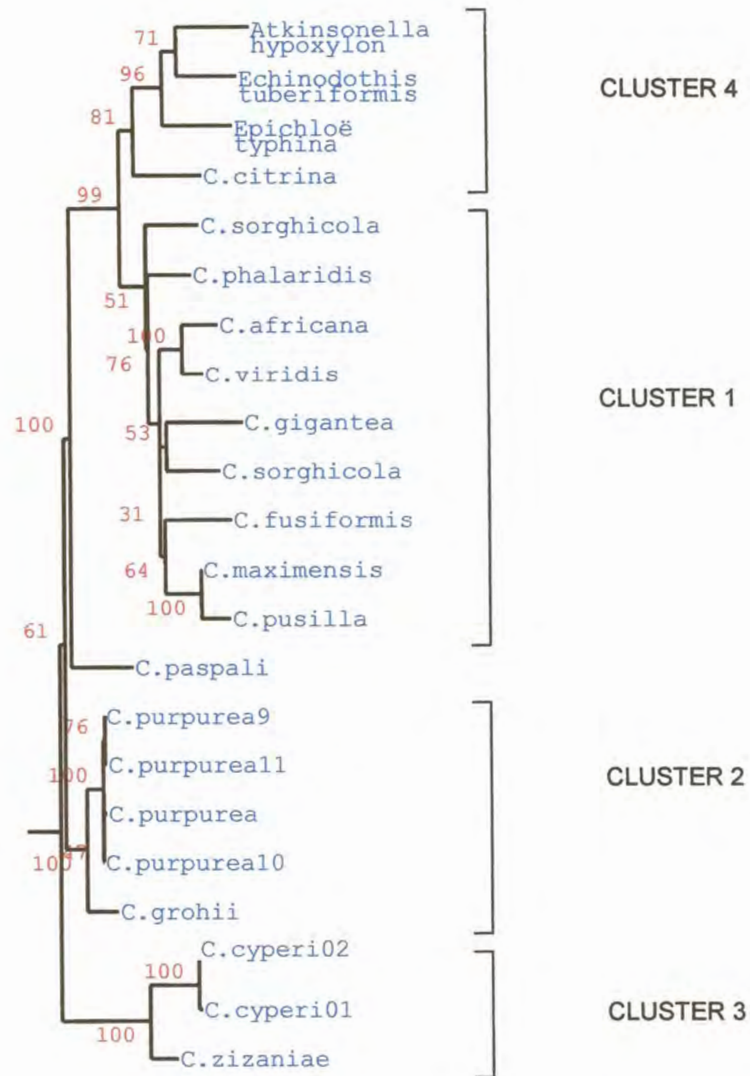


Figure 6.10 Phylogenetic tree showing phylogenetic relationships between *Claviceps* species and related teleomorphic species based on ITS1/2 spacers sequence data.

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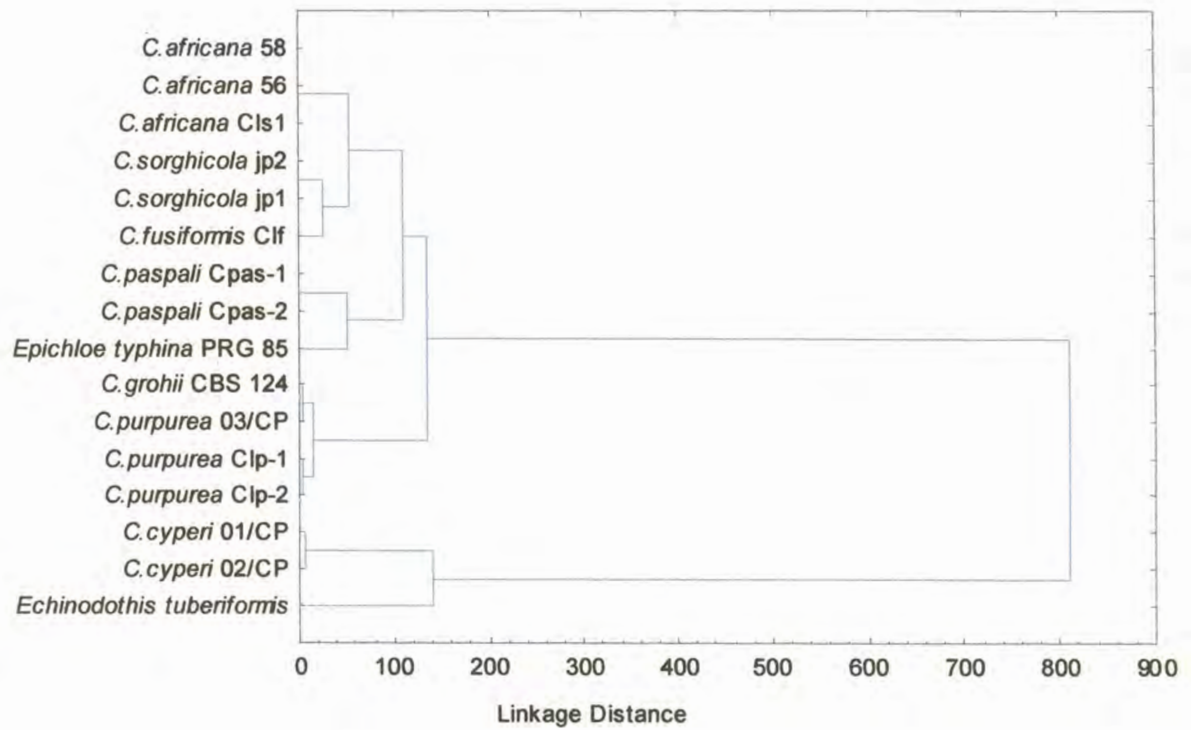
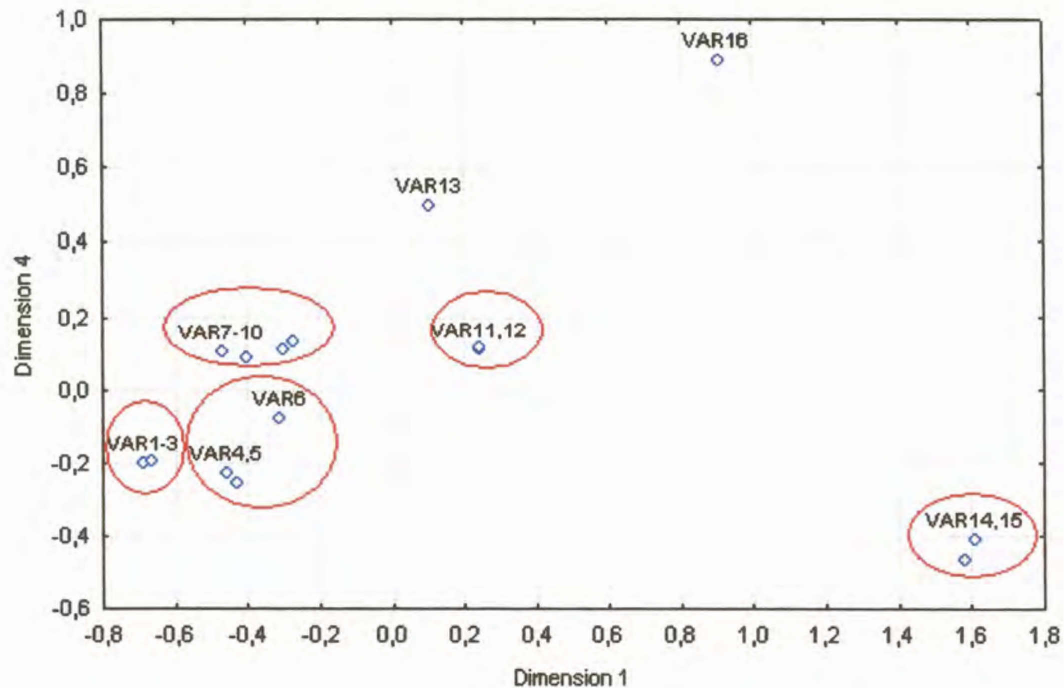


Figure 6.11 Dendrogram showing genetic distances between different *Claviceps* species and related teleomorphic species based on β-tubulin gene intron 3 region sequences.



Strain code:

VAR1	*C. africana*	Cls-1
VAR2	*C. africana*	Cla-56
VAR3	*C. africana*	Cla-58
VAR4	*C. sorghicola*	Cjap-1
VAR5	*C. sorghicola*	Cjap-2
VAR6	*C. fusiformis*	Clf -1
VAR7	*C. grohii*	CBS 124.47
VAR8	*C. purpurea*	03/CP
VAR9	*C. purpurea*	Clp-1
VAR10	*C. purpurea*	Clp-2
VAR11	*C. paspali*	Cpas-1
VAR12	*C. paspali*	Cpas-2
VAR13	*Epichloë typhina*	PRG; 85
VAR14	*C. cyperi*	01/CC
VAR15	*C. cyperi*	02/CC
VAR16	*Echinodothis tuberiformis*	B 351

Figure 6.12 Two-dimensional scatterplot showing genetic distances between different *Claviceps* species and related teleomorphic species based on β-tubulin gene intron 3 sequences.

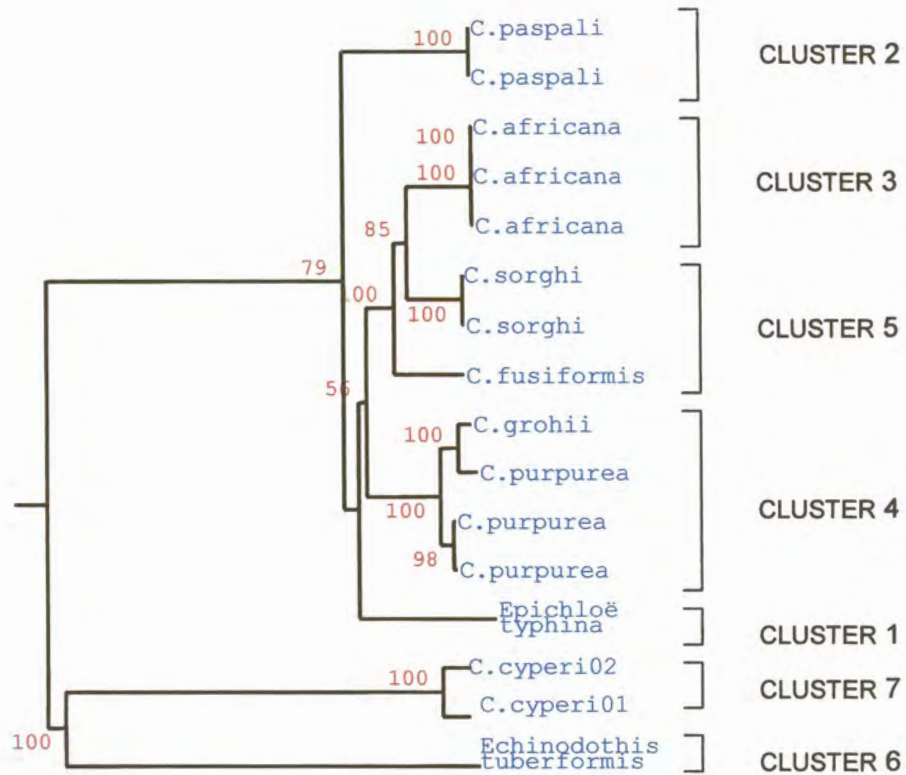


Figure 6.13 Phylogenetic tree showing phylogenetic relationships between *Claviceps* species and related teleomorphic species based on β-tubulin gene intron 3 region sequences.

Table 6.1 Strains of *Claviceps* species and related teleomorphic genera included in this study.

Species	Strain code	Date isolated	Locality	Host	Genbank accession no. for ITS1/2 spacers
C. cyperi	01/CC	2000	Greylingstad, SA	*Cyperus esculentus*	AY387492
C. cyperi	02/CC	2003	Greylingstad, SA	*Cyperus esculentus*	AY387491
C. purpurea	03/CP	2002	Onderstepoort, SA	*Festuca eliator*	AY387490
Tilletia indica	1325	2001			
C. fusiformis	SD 85			*Pennnisetum* sp.	AF133392
C. viridis	CBS 125.63	1963	India	*Oplismenus compositus*	AJ133404
C. grohii	CBS 124.47	1947	Canada	*Carex* sp.	AJ133395
C. zizaniae	CCM 8231	1996	Canada	*Zizania aquatica*	AJ133405
C. phalaridis	DAR 69619	1996	New South Wales, Austr	*Phalaris tuberosa*	AJ133399
C. pusilla	BRIP 26571	1996	Queensland, Australia	*Dicanthium aristatum*	AJ277544
C. sorghi		2000	India	*Sorghum bicolor*	AJ242869
C. sorghicola		1996	Japan;Kanto	*Sorghum bicolor*	AJ133397
C. paspali	MAL-1	1999	USA, Alabama	*Paspalum* sp.	AJ133398
C. purpurea	10	1996	Georgia, USA	*Dactylis glomerata*	U57669
C. purpurea	9 134 AU	1999	Victoria, Australia	*Phalaris tuberosa*	AJ133400
C. purpurea	11	1999		Strain Pepty 695/S	AJ133401
C. sulacata		1999	Brazil	*Brachiaria* sp.	AJ133403
C. citrina		1999	Mexico	*Distichlis spicata*	AJ133393
C. africana	NE1; Cafr	2000	Nebraska, USA	*Sorghum*	AF281176
C. viridis	CBS 125.63	1999	India	*Oplismenus compositus*	AJ133404
C. gigantea		1999	Mexico	*Zea mays*	AJ133394
C. maximensis	PM	1999	Paraguay	*Panicum maximum*	AJ133396
Atkinsonella hypoxylon		1996	Georgia, USA	*Danthonia spicata*	U57405
Echinodothis tuberiformis		1996	Alabama, USA	*Arundinaria tecta*	U57667
Epichloë typhina		2003			AB105952

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Species	Strain code	Date isolated	Locality	Host	Genbank accession no. for β-tubulin intron 3
C. cyperi	01/CP	2000	Greylingstad, SA	*Cyperus esculentus*	AY497005
C. cyperi	02/CP	2003	Greylingstad, SA	*Cyperus esculentus*	AY497775
C. grohii	CBS 124.47	1947	Canada	*Carex* sp.	AY438671
C. purpurea	Clp-1		Montana, USA	*Hordeum vulgare*	AF263567
C. purpurea	Clp-2		East Germany	*Secale cereale*	AF263568
C. purpurea	03/CP	2002	South Africa	*Festuca eliator*	AY438670
C. fusiformis	Clf-1		Africa	*Pennisetum typhoideum*	AF263569
C. paspali	Cpas-1	1998	North Carolina	*Paspalum* sp.	AF263605
C. paspali	Cpas-2	1997	Georgia	*Paspalum* sp.	AF263606
C. africana	Cis-1	1997	India	*Sorghum bicolor*	AF263596
C. africana	Cla-56	1998	Potchefstroom, SA	*Sorghum bicolor*	AF263591
C. africana	Cla-58	1998	Potchefstroom, SA	*Sorghum bicolor*	AF263592
C. sorghicola	Cjap-1	1989	Tochigi, Japan	*Sorghum bicolor*	AF263600
C. sorghicola	Cjap-2	1996	Tochigi, Japan	*Sorghum bicolor*	AF263601
Epichloë typhina	PRG; 85	1990		*Lolium perenne*	X52616
Echinodothis tuberiformis	B 351	1997			L78268

Table 6.2 Dendrogram and K-means clustering of South African isolates of *Claviceps purpurea*, *C. grohii*, *C. cyperi* and *Tilletia indica* based on multilocus fingerprinting.

Euclidean distances between clusters
Distances below diagonal

	No. 1	No. 2
No. 1	0	
No. 2	46.13407135	0

Distance from respective cluster centre *

	Variable	Cluster	Distance *
C. grohii CBS 124.47	1	2	15.5083758
C. purpurea 03/CP	2	2	15.5083758
T. indica 1325	3		
C.cyperi 01/CC	4	1	1.815852417
C.cyperi 02/CC	5	1	1.815852417

MANOVA discriminant analysis for BOX A1R; ERIC2 and ARP-7 multilocus primers

DISCR_01	EIGV= 62.3%	L= 0.0001	*P*<=0.001%
DISCR_02	EIGV= 37.7%	L= 0.0154	*P*<=0.001%

Similarity matrix

	C. grohii CBS	*C. purpurea*	*T. indica*	*C. c 01/CC*	*C. c 02/CC*
***C.grohii* CBS 124.47**	100	52.65	43.61	41.56	46.18
***C.purpurea* 03/CP**	52.65	100	52.2	34.09	32.16
T.indica	43.61	52.2	100	20.92	22.85
***C.cyperi* 01/CC**	41.56	34.09	20.92	100	95.69
***C.cyperi* 02/CC**	46.18	32.16	22.85	95.69	100

Table 6.3 Dendrogram and K-means clustering of different *Claviceps* species and related teleomorphic species showing genetic distances based on ITS1/2 spacers sequence data.

Euclidean distances between clusters
Distances below diagonal

	No. 1	No. 2	No. 3	No. 4
No. 1	0			
No. 2	8.539524078	0		
No. 3	12.09759808	9.195717812	0	
No. 4	6.131327629	8.274378777	10.75427914	0

Distance from respective cluster centre *

	Variable	Cluster	Distance *
C. africana	1	1	2.142656396
C. viridis	2	1	1.702083295
C. gigantea	3	1	3.271944819
C. sorghi	4	1	2.24644875
C. fusiformis	5	1	2.646069444
C. maximensis	6	1	2.716711594
C. pusilla	7	1	2.418075173
C. phalaridis	8	1	2.817050658
C. sorghicola	9	1	2.530274602
Atkinsonella hypoxylon	10	4	2.568753456
Echinodothis tuberiformis	11	4	2.691838464
Epichloë typhina	12	4	2.391574938
C. citrina	13	4	3.727855068
C. cyperi 01/CC	14	3	0.836599659
C. cyperi 02/CC	15	3	1.022795733
C. zizaniae	16	3	1.837172289
C. purpurea 9	17	2	1.250676585
C. purpurea 11	18	2	1.678548555
C. purpurea 03/CP	19	2	1.014416287
C. purpurea 10	20	2	1.17838273
C. grohii	21	2	1.403063027
C. paspali	22	2	3.144202618

Multidimensional scaling: final configuration

No. of dimensions = 6
Measure of goodness-of-fit: Stress values

D-star:Raw stress	D-hat:Raw stress	Alienation	Stress
0.023605	0.0088243	0.0069836	0.0042699

Table 6.4 Dendrogram and K-means clustering of different *Claviceps* species and related teleomorphic species showing genetic distances based on β-tubulin gene intron 3 region sequence data.

Euclidean distances between clusters
Distances below diagonal

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
No. 1	0						
No. 2	9.537975311	0					
No. 3	10.74645329	11.3850803	0				
No. 4	9.716282845	11.5703497	10.1820211	0			
No. 5	9.56938076	9.92785358	5.14885426	8.502935	0		
No. 6	46.33493423	47.7928696	51.6772575	51.13251	50.81779099	0	
No. 7	48.63568497	49.4319077	53.3657837	52.53014	52.49116898	26.51585	0

Distance from respective cluster centre *

	Variable	Cluster	Distance*
C. africana Cls-1	1	3	0.06454972
C. africana Cla-56	2	3	0.06454972
C. africana Cla-58	3	3	0.12909944
C. sorghicola Cjap-1	4	5	1.53769759
C. sorghicola Cjap-2	5	5	1.52661572
C. fusiformis Clf-1	6	5	3.06055777
C. grohii CBS124.47	7	4	0.98862673
C. purpurea 03/CP	8	4	1.37541187
C. purpurea Clp-1	9	4	1.16479947
C. purpurea Clp-2	10	4	1.01621617
C. paspali Cpas-1	11	2	0.073951
C. paspali Cpas-2	12	2	0.073951
Epichloe typhina	13	1	0
C. cyperi 01/CC	14	7	0.77237459
C. cyperi 02/CC	15	7	0.77237459
Echinodothis tuberiformis	16	6	0

Multidimensional scaling: final configuration

No. of dimensions = 6
Measure of goodness-of-fit: Stress values

D-star:Raw stress	D-hat:Raw stress	Alienation	Stress
0.0000033	0.000001	0.00001139	0.00000624

7. GENERAL DISCUSSION

The previous chapters have provided descriptions of the symptoms of ergot on yellow nut sedge (*Cyperus esculentus* L.) and of live specimens of the causal organism, *Claviceps cyperi* Loveless. Evidence was also presented regarding the phylogeny and alkaloid profile of the pathogen, the morphology, taxonomy, physiology and mode of infection of its anamorph, and some aspects pertaining to the epidemiology and ecology of the disease. Although many aspects have been elucidated in the study, some questions remained unanswered or unclear, the most notable being the taxonomic position of the pathogen and the origin, distribution and management of the disease.

Phylogenetically, *C. cyperi* proved to be a distinct species, but closely related to *Claviceps zizaniae* (Fyles) Pantidou, with which it also shared a common alkaloid profile. However, the presence of the extended ITS regions unique to these two species observed in the present study may explain their outgroup placement insufficiently. Sequence data derived from ITS regions should be interpreted with caution because not all copies of these regions are identical in the same strain. Several strains of different species within a genus need to be screened to ensure that only copy type is amplified and sequenced. Multiple intron-rich protein coding genes should be sequenced as well, and compared with existing sequence data in GenBank. These protein-coding genes evolve faster than the ITS regions and are able to discriminate between closely related phylogenetic cryptic species within a morphological species (Taylor *et al.* 2000). O'Donnell & Cigelnik (1997) have demonstrated the potential of intron-rich protein-coding genes as species markers for *Fusarium*, and probably other fungal genera as well. According to Geiser *et al.* (1998) and Carbone & Kohn (2001), introns flanked by the more conserved exons evolve at a higher rate than ITS regions, hence increasing the resolving power of these genes to reveal variation at intraspecific level. Determination of the genealogies of these genes within the genus *Claviceps* may also prove rewarding with regard to the overall phylogeny of the genus and determining a common ancestor or ancestors for different species. The ongoing process of speciation within the genus *Claviceps* in association with different hosts, as well as the geographic origin of different species may also be established using appropriate species markers and the concept of Genealogical Concordance Phylogenetic Species Recognition.

The apparent holoblastic conidiogenesis observed in the spacelial state of *C. cyperi* in this study was a matter of concern as it implied a revision of the genus *Sphacelia*. Very recently, however, Pažoutová *et al.* (2004) showed that conidiation in *Claviceps* is pleomorphic, with conidiogenesis in *C. zizaniae* and *Claviceps citrina* Pažoutová, Fucík., Leyva-Mir & Flieger being ephelidial (holoblastic and sympodial), typical of the genus *Ephelis*. Although the existence of holoblastic conidiation in *Claviceps* would have resolved the taxonomy of *C. cyperi*, and placed its anamorph in *Ephelis*, this could not be done as the conidia of *Ephelis* species are produced sympodially and often form whorls consisting of 3 to 8 spores (Rykard *et al.* 1984; White 1997), whereas *C. cyperi* produces conidia singly and certainly not in sympodial succession. The issue is further compounded by the claim of Pažoutová *et al.* (2004) that both macro- and microconidia have been observed in all studied phialidic *Claviceps* species except *C. citrina* and *C. purpurea* (Fr.:Fr.) Tul., though microconidia could not be discerned in honeydew or cultures of *C. cyperi*. The confusion surrounding the taxonomic status of the nut sedge ergot pathogen therefore remains.

From an ecological perspective the most perplexing question obviously is the apparent confinement of *C. cyperi* to South Africa and its disappearance for more than 50 years since first recorded from in and around Pretoria during World War II until the incidents of ergotism associated with the intake of ergotised nut sedge in 1996/1997 at Greylingstad, Memel and Vrede. Despite concerted efforts the past eight years, the disease could not be detected in the Pretoria/Kempton Park area. It should nevertheless be noted that Greylingstad, Memel and Vrede do not feature in Doidge (1950) and the possibility therefore exist that these sites have never been visited in previous disease surveys. There is, however, a reference in Doidge (1950) to a *Cerebella* species on *Cyperus* from Mount Edgecombe in KwaZulu-Natal. Examination of the specimen, which was collected in 1945, indicated that the host was *Cyperus rotundus* L. and indeed colonised by *Cerebella*. Although no sign of ergot could be found, the presence of *Cerebella* is a sure indication of previous infection with a species of *Claviceps* (Langdon 1942; Loveless 1964). It is thus possible that *C. cyperi* has been present in other areas of South Africa, but remained unnoticed due to the inconspicuousness of the symptoms and the fact that it was not involved in any reported incident of ergotism. The recent sporadic eruptions can probably be ascribed to a combination of the following: (i) changes in tillage practices, (ii)

conducive climate, (iii) invasion by, or an increase in populations of spotted maize beetle (*Astylus atromaculatus* Blanchard) and the unidentified thrips species associated with the disease, and (iv) the conversion to forced crib-feeding by the farmers concerned.

Deep ploughing is an effective means of eliminating or confining ergot sclerotia (Bandyopadhyay *et al.* 1998; Bhuiyan *et al.* 2002). This practice has, since about 1980, increasingly been replaced by no-tillage or alternatively by shallow ripping and discing in the affected areas, and could therefore have resulted in a gradual build-up of inoculum of *C. cyperi.* Tillage is also an essential component in nut sedge control (Hauser 1962; Glaze 1987), and the reduction in mechanical cultivation necessitated the use of herbicides, which not only does not provide total control, but possibly could have predisposed the weed to ergot infection (Altman & Campbell 1977). The first outbreak of ergotism in 1996 was preceded by an exceptionally cold winter followed by high rainfall in spring and early summer, a climatological pattern which incidentally also prevailed during the latter part of World War II (SA Weather Services). Cold weather followed by rain and high humidity is conducive to the germination of ergot sclerotia (Brentzel 1947; Eleutherius & Meyers 1974). The ensuing inoculum propably was disseminated by insects such as thrips and spotted maize beetle of which the numbers increased because indiscriminate spraying of crops destined for fodder with insecticides obviously is undesirable.

The main reason for the outbreaks of ergotism undoubtedly is the practice of forced crib-feeding. When allowed to graze ad-lib it is highly unlikely that cattle would consume nut sedge plants, particularly when they are ergotised and the honeydew colonised by a trichotecene-producing fungus such as *Fusarium heterosporum* Nees, as in the present study. Crib-feeding is in any case not an effective farming practice and leads to various metabolic and reproductive disorders, most notably acidosis. Unfortunately dairy farmers in the new South Africa are compelled to resort to this practice for financial and security reasons such as AgriBEE, accompanied by deregulation of the agricultural sector, abolition of subsidies, land restitution, dwindling research funding and capacity, and the ever-increasing incidence of stock theft, and maiming farm attacks.

Fortunately, it is not the end of the road for the farmers affected. World sales of Parlodel, the dopaminergic agent of which the active ingredient 2-bromo-α-ergocryptine, is derived

from α-ergocryptine, the main alkaloid produced by *C. cyperi*, amounts to approximately \$143 million (± R 860 million) per annum. This represents an annual usage of 247 kg of 2-bromo-α-ergocryptine, containing about 222 kg of α-ergocryptine. Yellow nut sedge produces on average 600 000 inflorescences per hectare (Hill *et al.* 1963). When ergotised, the mean number of sclerotia per inflorescence is 10 (Chapter 4), each between 8—15 x 1.5 mm in size (Chapter 2), with a mean volume of 6.5 μl and mean α-ergocryptine content of 3600 mg l^{-1} (Chapter 5). This implies that one hectare of ergotised nut sedge yields about 140 g of the alkaloid to the value of R 436 842. Even when accepted that the producer's price of sclerotia would probably be only 10 % of that of the final product, it still means a profit of more than R 40 000 per hectare after harvesting. To supply the world demand for Parlodel would require about 1 600 ha of ergotised nut sedge. Synthetic production of α-ergocryptine with species such as *C. zizaniae* (and *C. cyperi* if it can be induced to produce the alkaloid in culture) could add R 11 to R 70 g^{-1} to the production cost. The cost of producing α-ergocryptine from wheat ergotised by *C. purpurea*, the present means of commercial production, is not known but is likely to be relatively high due to the low α-ergocryptine yield of *C. purpurea* and the added expenditure of growing, infecting and maintaining the wheat.

As indicated in the first paragraph, facets of the nut sedge ergot complex that still need to be clarified include the molecular systematics of *C. cyperi* and the taxonomic position of the anamorph, as well as the distribution and dissemination of the disease. Negotiations are presently underway to obtain material of the ergotised specimens of *Cyperus latifolius* Poir. and *Cyperus rigidifolius* Steud. maintained at the International Mycological Institute for inclusion in molecular analysis with new and existing isolates of *C. cyperi*. The above two *Cyperus* species also occur in South Africa (Gordon-Gray 1995) and are morphologically closely related to *C. esculentus* and *C. rotundus*. It should therefore be worthwhile to conduct a survey of these and other related and common unrelated *Cyperus* species in South Africa for ergot infection. Lastly, a collaborative study is planned with a specialist entomologist to elucidate the involvement of the thrips species and spotted maize beetle, the latter which incidentally also produces a toxin that can be lethal when large numbers of the beetle are ensilaged in fodder fed to cattle (Drinkwater 1997), in the epidemiology of nut sedge ergot and pathology of ergotism.

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