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

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.

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.

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

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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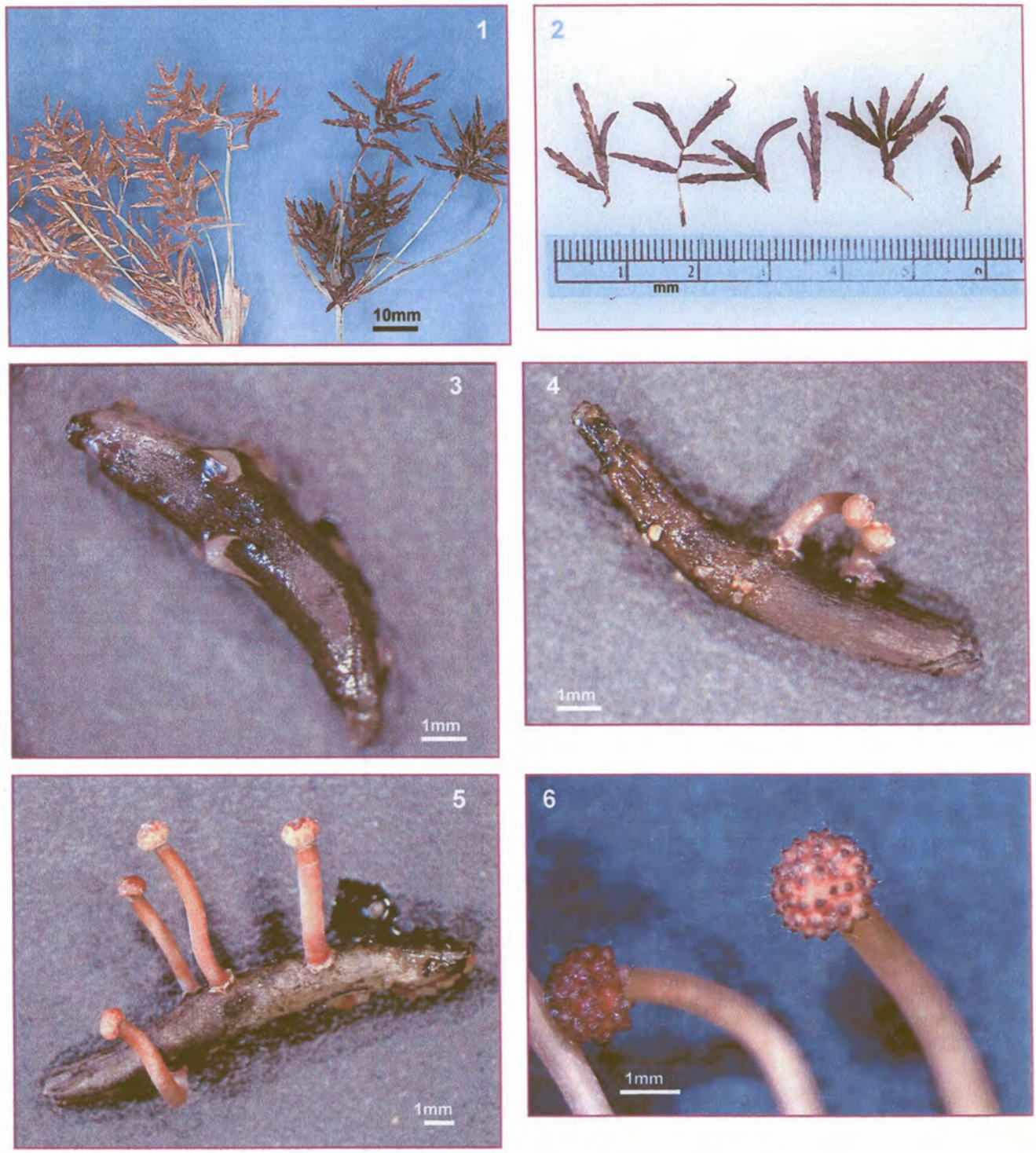
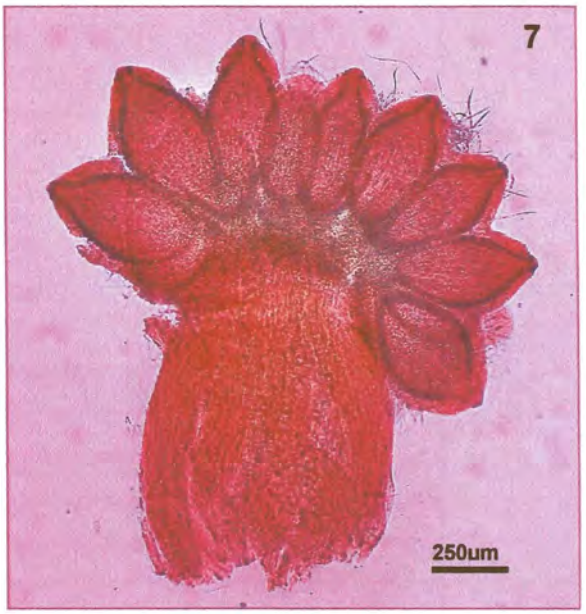


Figure 2.1 ~~Healthy and ergotised inflorescences of *Cyperus esculentus*.~~
Figure 2.2 *Cyperus esculentus* inflorescences containing sclerotia of *Claviceps cyperi*.
Figures 2.3-2.6 Germination process of sclerotia of *Claviceps cyperi*.
2.3 First stage of germination of sclerotium.
2.4 Two stromata 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).



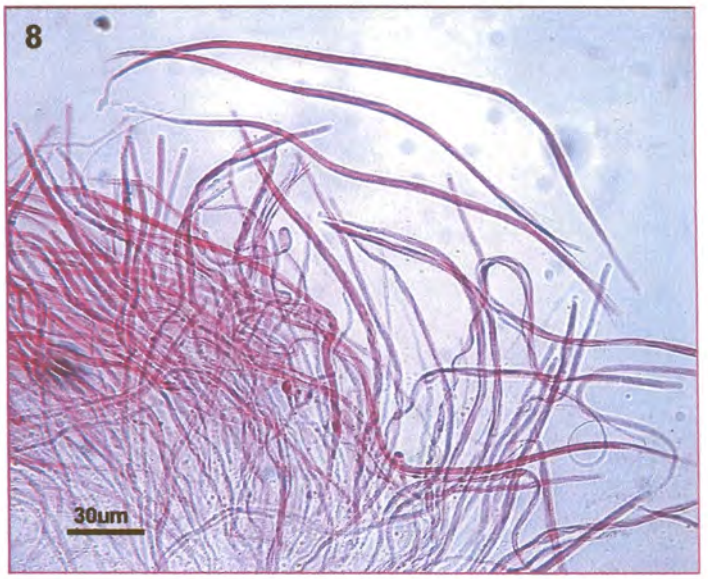


Figure 2.7 Hand-cut section through capitulum showing perithecia of *Claviceps cyperi*.
Figure 2.8 Asci of *Claviceps cyperi* with filiform ascospores.

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 %.

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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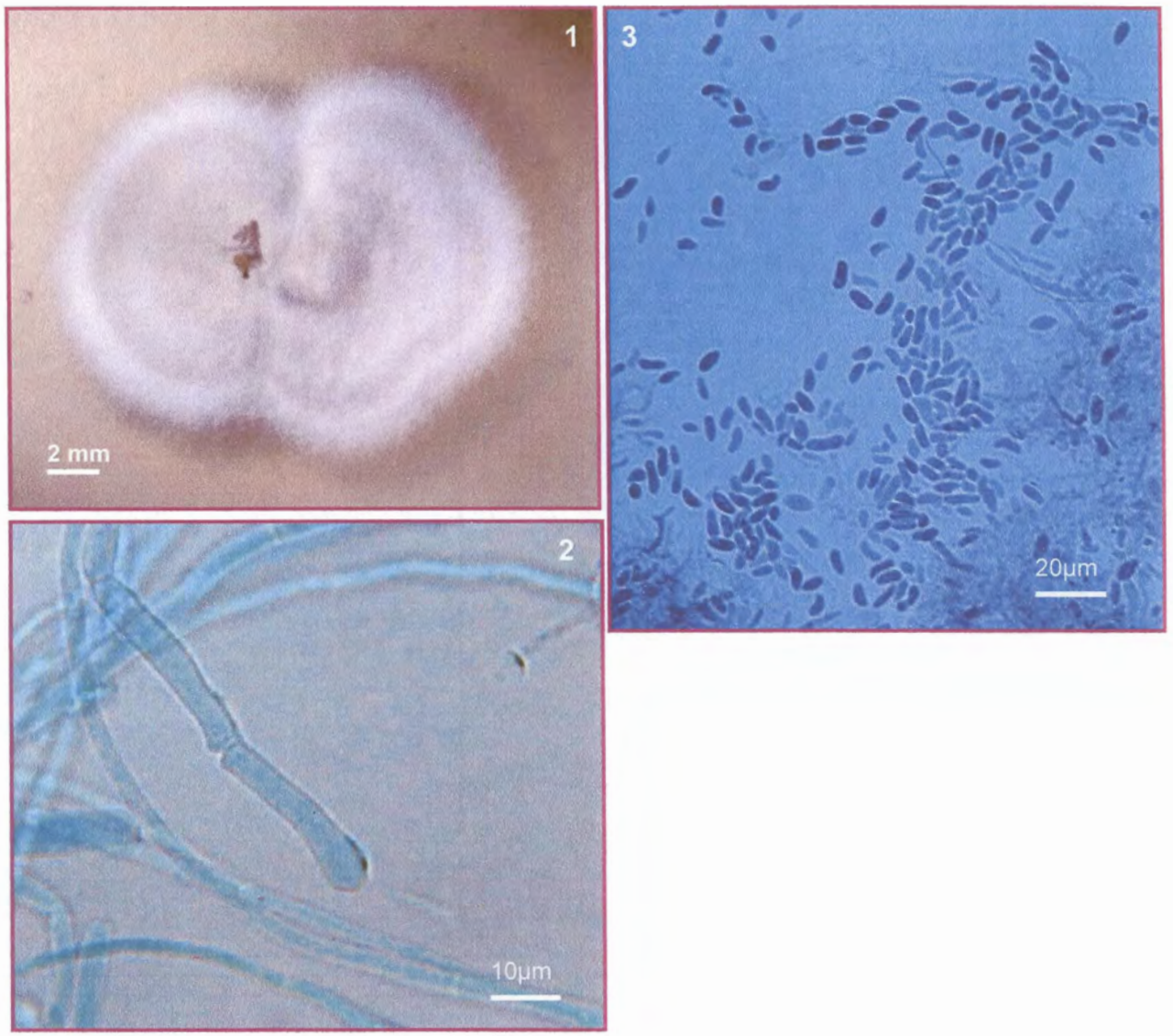
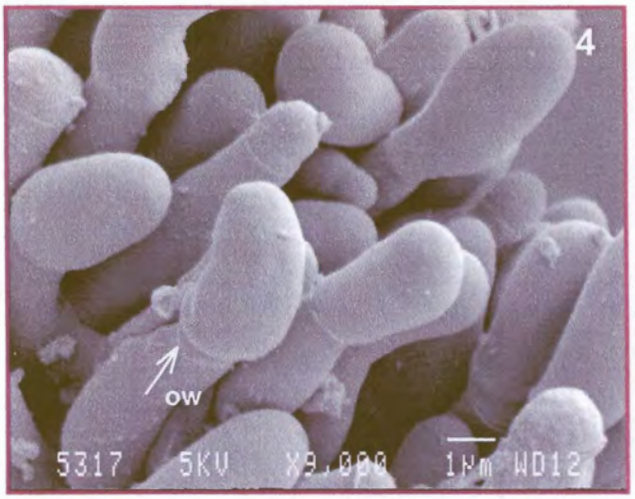
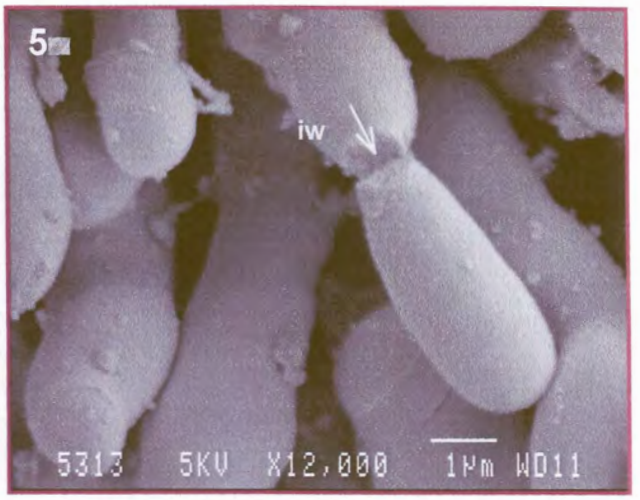
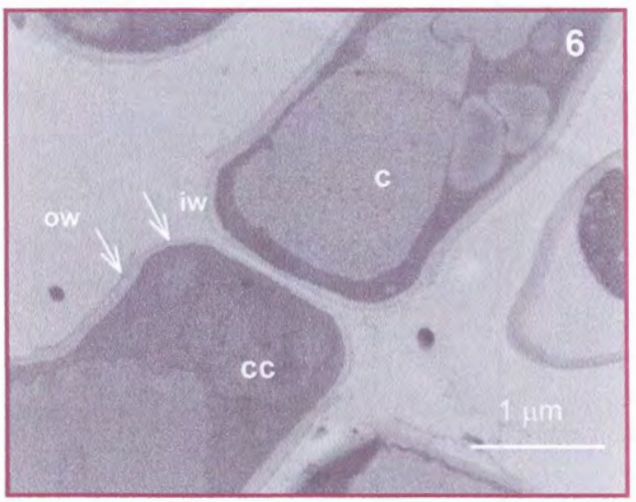


Figure 3.1 Culture of the *Sphacelia* state of *Claviceps cyperi* on potato-dextrose agar after 2 weeks.
Figure 3.2 Enlarged cells observed in hyphae of the *Sphacelia* state of *Claviceps cyperi*.
Figure 3.3 Conidia of the *Sphacelia* state of *Claviceps cyperi* produced on 2 % malt extract agar.







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

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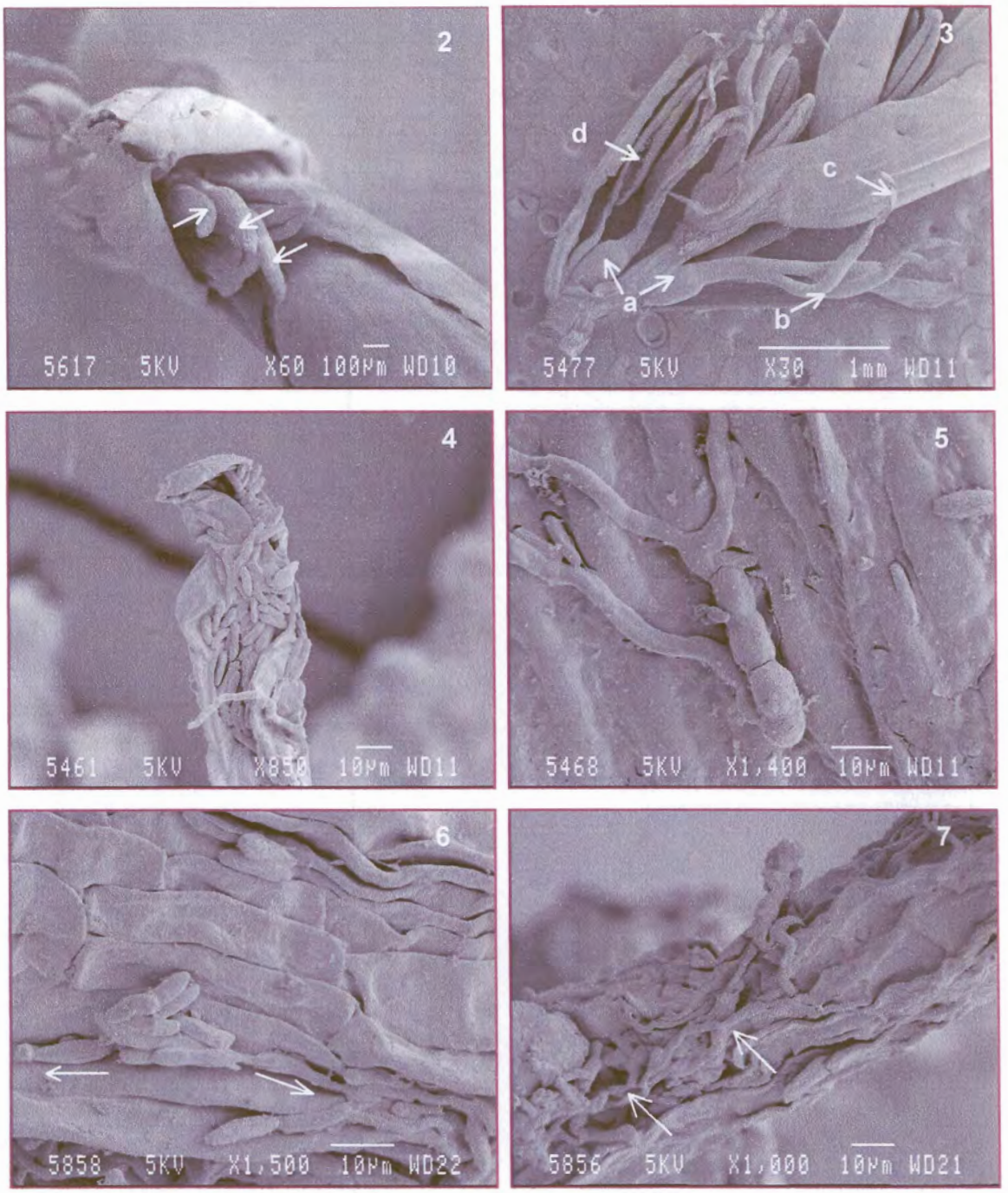
Figure 4.1 Inflorescence of *Cyperus esculentus* infected with *Claviceps cyperi*. Drops of honeydew, as well as black layers formed by *Cladosporium cladosporioides* are clearly visible.



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

4.2 Young stylodia (arrows) protruding through glume opening.

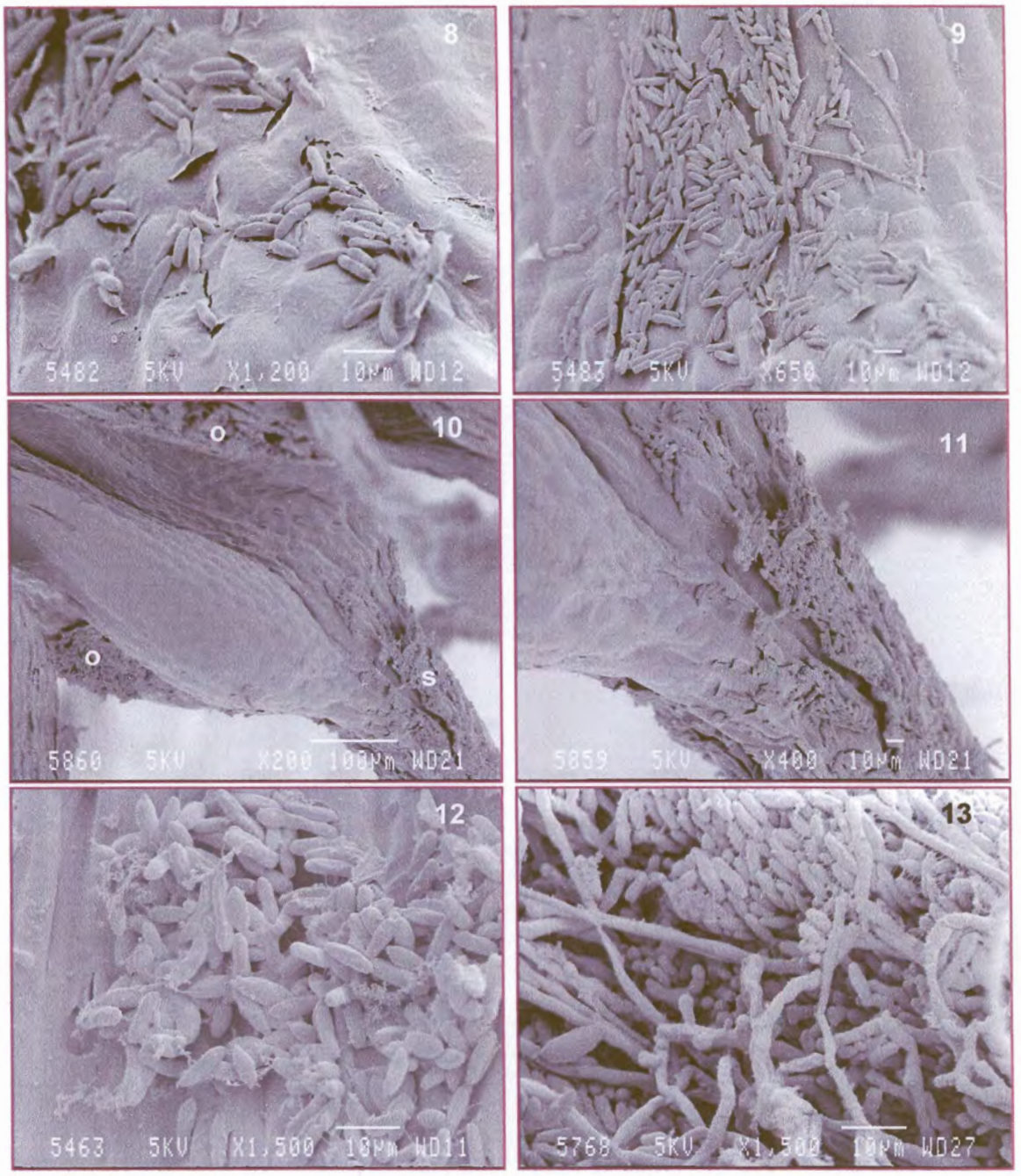
4.3 Morphology of the pistil and stamen: (a) ovary, (b) stylodium, (c) stigma, (d) anther.

4.4 Stigma infected superficially by conidia.

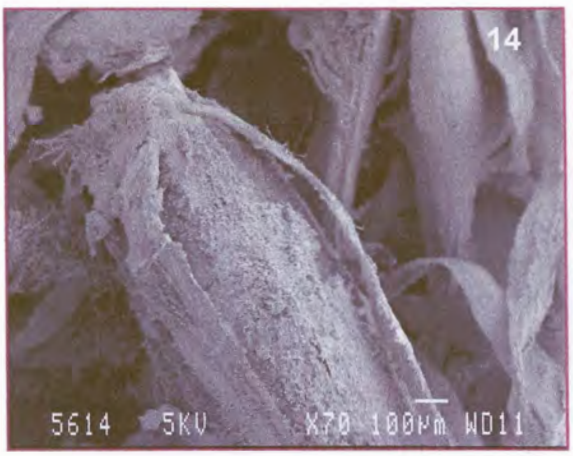
4.5 Conidium forming germ tubes on the stylodium and spreading.

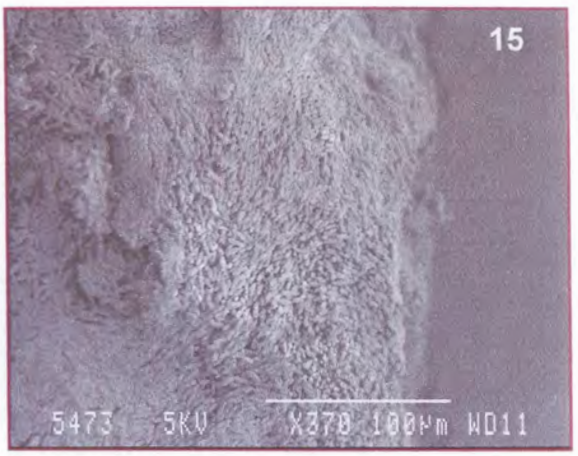
4.6 Mycelium spreading along length of stylodium (arrows).

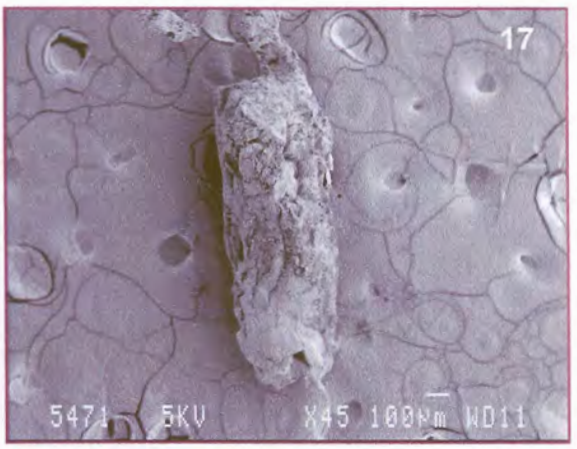
4.7 Conidia and mycelium clearly visible on base of style (arrows).

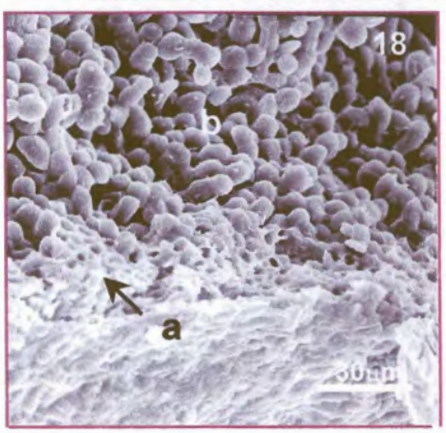


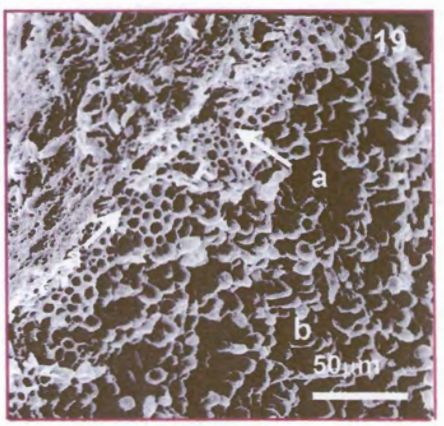
Figures 4.8-4.13 SEM micrographs of infection of *Cyperus esculentus* with *Claviceps cyperi*:
4.8 Conidia on apical part of ovary.
4.9 Hyphae spreading over rest of ovary.
4.10 Base of stylodium (s) and ovary (o) infected with conidia.
4.11 Closer view of base of stylodium and ovary neck with conidia.
4.12 Conidial mass starting to form.
4.13 Conidial mass with interspersed hyphae.







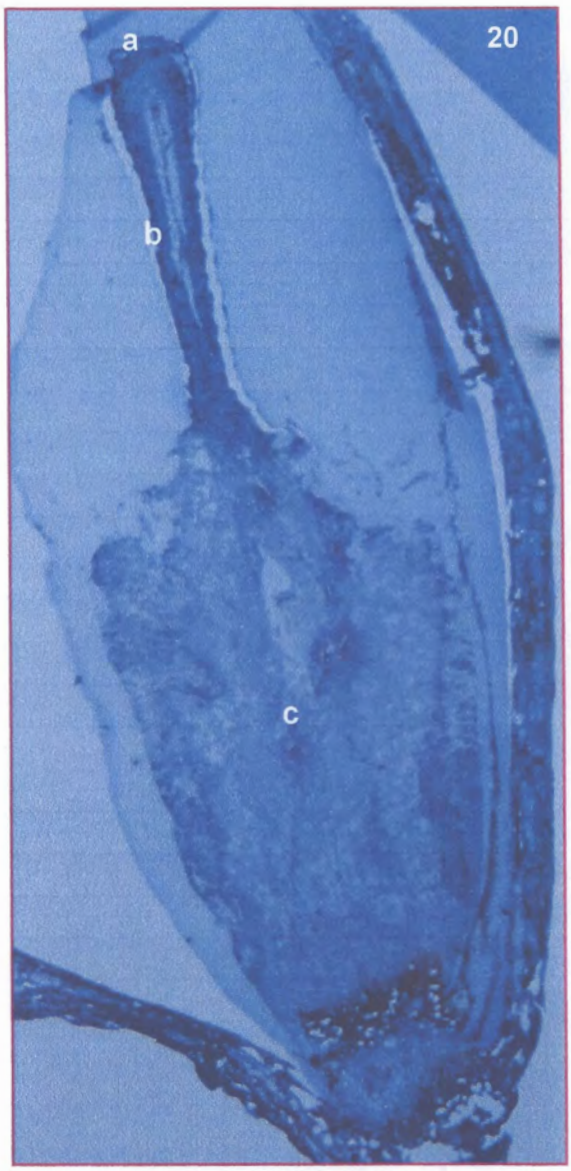


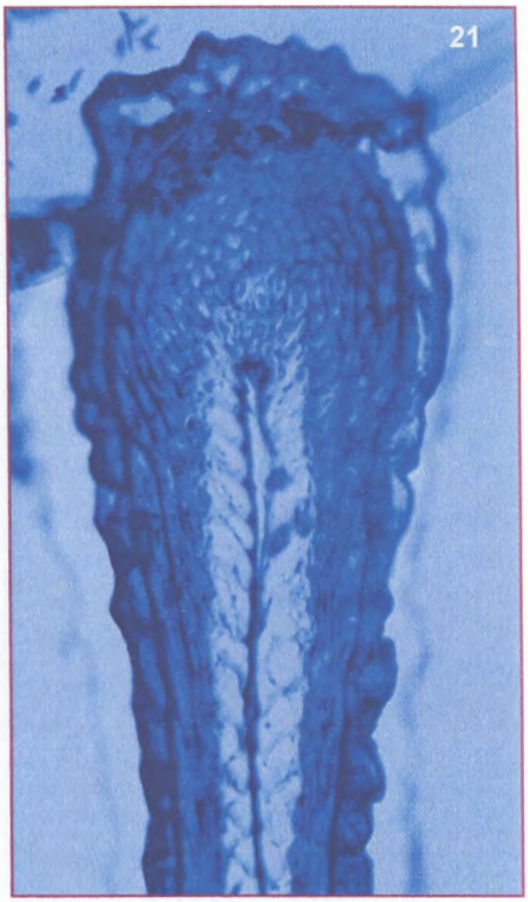


Figures 4.14-4.17 SEM micrographs of infection of *Cyperus esculentus* with *Claviceps cyperi:*

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.

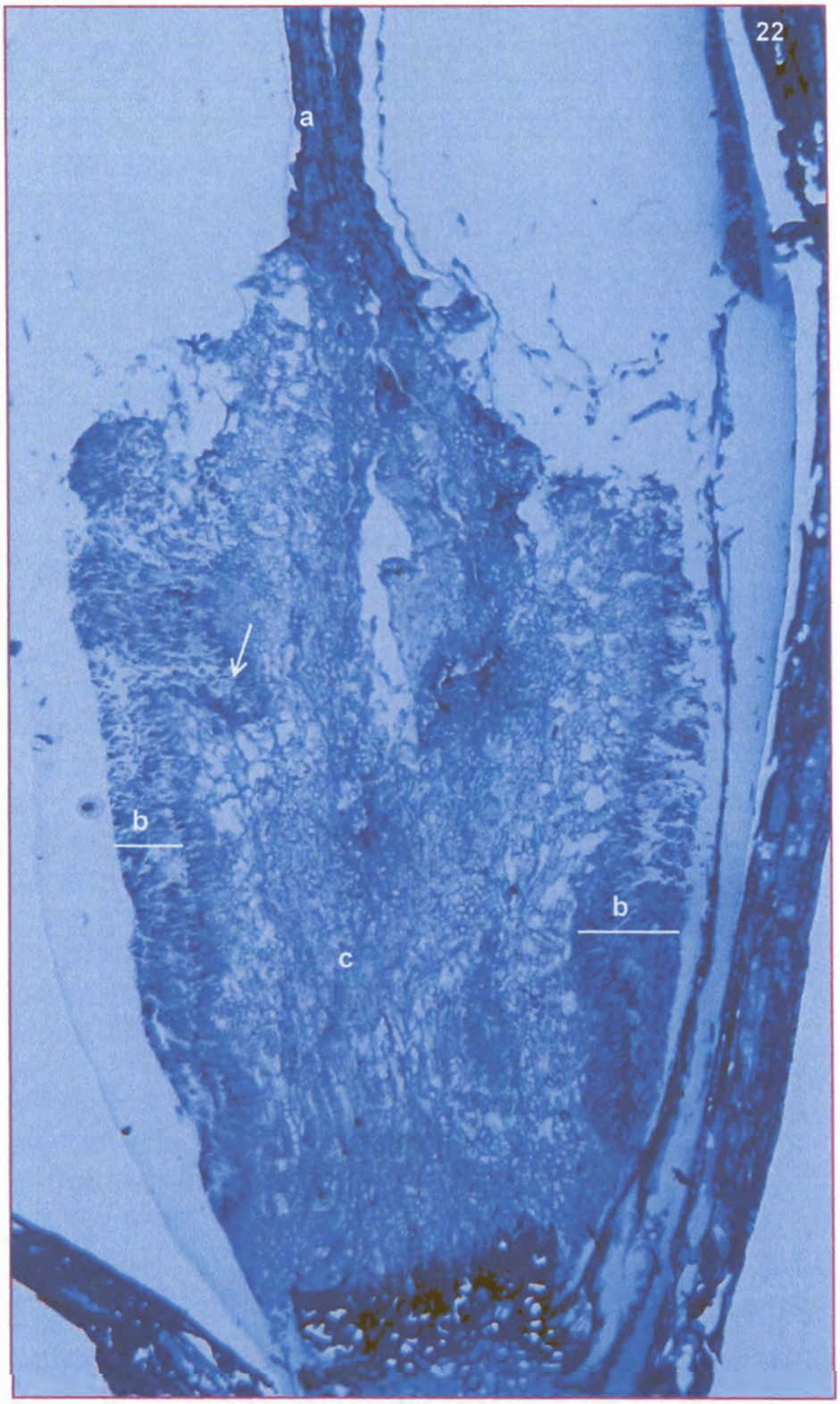


Figure 4.22 Section through pistil of *Cyperus esculentus* infected with *Claviceps cyperi*: (a) stylodium (no obvious infection), (b) conidial layer covering outside of ovary and 'labyrinthine chambers' starting to develop (arrow), and (c) hyphal cells filling ovary.

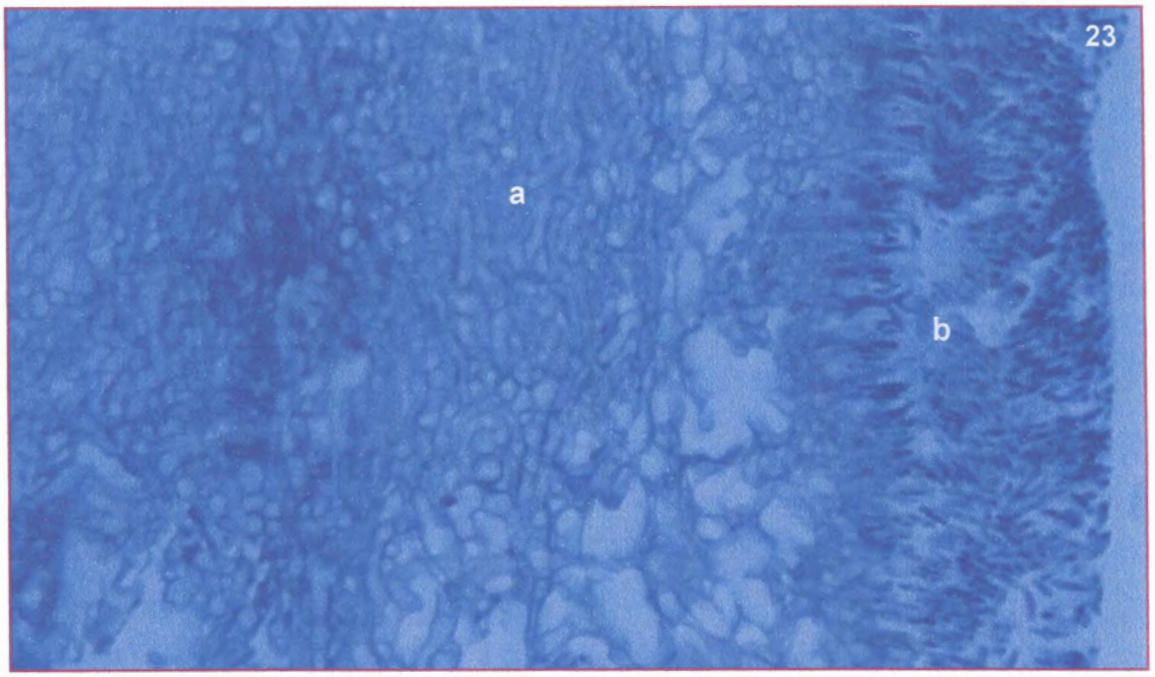
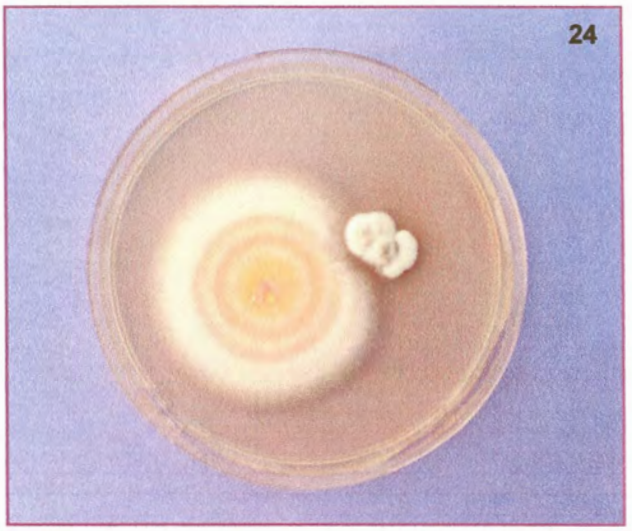
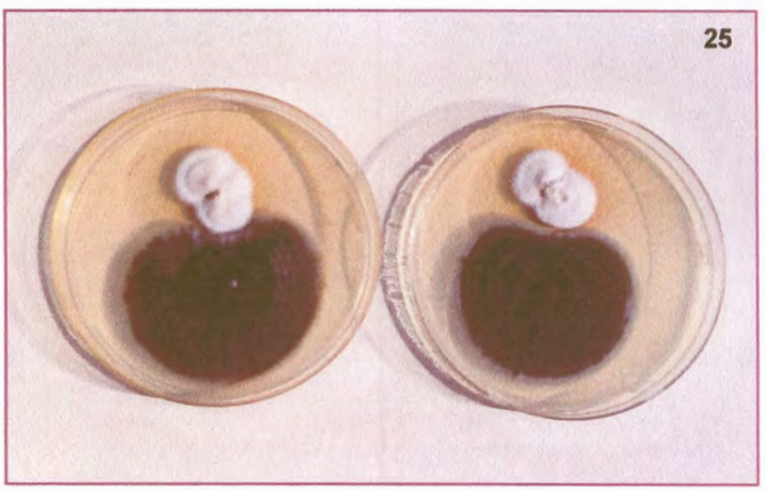


Figure 4.23 Closer view of section through ovary of *Cyperus esculentus* infected with *Claviceps cyperi*: (a) hyphal cells visible on the inside and (b) conidial layer covering ovary on the outside.





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

4.24 *Fusarium heterosporum*.

4.25 *Cladosporium cladosporioides*.