

ERGOT OF NUT SEDGE IN SOUTH AFRICA

Ella Johanna van der Linde

**A thesis submitted in partial fulfillment of the requirements for the
degree of**

PHILOSOPHIAE DOCTOR (PLANT PATHOLOGY)

in the

**FACULTY OF NATURAL AND AGRICULTURAL SCIENCES
UNIVERSITY OF PRETORIA**

July 2005



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

ACKNOWLEDGEMENTS

I wish to express my appreciation to the following organisations and persons who made this thesis possible:

1. The Plant Protection Research Institute (PPRI), Agricultural Research Council (ARC), for financial support and use of its facilities during the course of the study.
2. The following persons are gratefully acknowledged for their assistance during the course of the study:
 - Prof Theuns W. Naude, formerly of ARC Onderstepoort Veterinary Institute.
 - Prof Christo Botha, University of Pretoria, Onderstepoort.
 - Quenton Kritzinger, University of Pretoria.
 - Prof. George Rottinghaus, University of Missouri, Columbia, U.S.A.
 - Dr Wilhelm Botha and Dr Isabel Rong, ARC-PPRI.
 - Oloff O'Brien, Sarie Velthuisen, Flip van der Merwe, Hendrik van Tonder and Elsa van Niekerk, ARC-PPRI, for technical assistance.
3. Prof F.C. Wehner, my supervisor and Prof T.A.S. Aveling, my co-supervisor, for their guidance and support.
4. Family, friends and colleagues for their encouragement and support.

ERGOT OF NUT SEDGE IN SOUTH AFRICA

by

ELLA JOHANNA VAN DER LINDE

SUPERVISOR Prof F.C. Wehner
CO-SUPERVISOR Prof T.A.S. Aveling
DEPARTMENT Microbiology and Plant Pathology
DEGREE Ph.D.

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 β -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.

LIST OF FIGURES

Figure 1.1:	Holstein cows salivating with mouths open and tongue protruding (Photo: T.W. Naude).	7
Figure 1.2:	Holstein cows wading into water to cool off, suffering from hyperthermia (Photo: T.W. Naude).	7
Figure 1.3:	Development of winter coat, black parts turning brown.	7
Figure 1.4:	Yellow nut sedge infestation of a maize field.	7
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.	8
Figure 2.1:	Healthy and ergotised inflorescences of <i>Cyperus esculentus</i> .	19
Figure 2.2:	Closer view of <i>Cyperus esculentus</i> inflorescences containing sclerotia of <i>Claviceps cyperi</i> .	19
Figure 2.3:	First stage of germination of sclerotium.	19
Figure 2.4:	Two stromata emerging.	19
Figure 2.5:	Four stromata with stipes elongated and capitula almost mature.	19
Figure 2.6:	Mature capitulum with individual perithecia visible – asci protruding through ostioles (arrow).	19
Figure 2.7:	Hand-cut section through capitulum showing perithecia of <i>Claviceps cyperi</i> .	20
Figure 2.8:	Asci of <i>Claviceps cyperi</i> with filiform ascospores.	20
Figure 3.1:	Culture of the <i>Sphacelia</i> state of <i>Claviceps cyperi</i> on potato-dextrose agar after 2 weeks.	32
Figure 3.2:	Enlarged cells observed in hyphae of the <i>Sphacelia</i> state of <i>Claviceps cyperi</i> .	32
Figure 3.3:	Conidia of the <i>Sphacelia</i> state of <i>Claviceps cyperi</i> produced on 2 % malt extract agar.	32
Figure 3.4:	Conidiogenous cells producing conidia (SEM micrograph).	33
Figure 3.5:	Conidium seceding from conidiogenous cell (SEM micrograph).	33
Figure 3.6:	Section through conidiogenous cell (cc) and conidium (c) with arrows indicating inner wall (iw) and outer wall (ow) (TEM	

	micrograph).	33
Figure 4.1:	Inflorescence of <i>Cyperus esculentus</i> infected with <i>Claviceps cyperi</i> . Drops of honeydew, as well as black layers formed by <i>Cladosporium cladosporioides</i> are clearly visible.	49
Figure 4.2:	Young stylodia (arrows) protruding through glume opening as indicated by arrows.	50
Figure 4.3:	Morphology of the pistil and stamen: (a) ovary, (b) stylodium, (c) stigma, (d) anther.	50
Figure 4.4:	Stigma infected with conidia.	50
Figure 4.5:	Conidium forming germ tubes on the stylodium and spreading.	50
Figure 4.6:	Mycelium spreading along length of stylodium (arrows).	50
Figure 4.7:	Conidia and mycelium clearly visible on base of style (arrows).	50
Figure 4.8:	Conidia on apical part of ovary.	51
Figure 4.9:	Hyphae spreading over rest of ovary.	51
Figure 4.10:	Base of stylodium (s) and ovary (o) infected with conidia.	51
Figure 4.11:	Closer view of base of stylodium and ovary neck with conidia.	51
Figure 4.12:	Conidial mass starting to form.	51
Figure 4.13:	Conidial mass with interspersed hyphae.	51
Figure 4.14:	Ovary totally covered with conidia.	52
Figure 4.15:	Closer view of conidial mass.	52
Figure 4.16:	Sclerotium beginning to develop with pollen (arrow) visible.	52
Figure 4.17:	Ovary completely deformed and covered with conidia with base of withered style visible at the top.	52
Figure 4.18:	Sections through sclerotia of <i>Claviceps cyperi</i> showing difference between outer layer (a) and inner layers containing bundles of longer hyphae (b).	52
Figure 4.20:	Pistil consisting of (a) stigma, (b) stylodium, (c) ovary.	53
Figure 4.21:	Stigma and stylodium with no infection evident, although ovary already infected.	53
Figure 4.22:	Section through pistil of <i>Cyperus esculentus</i> infected with <i>Claviceps cyperi</i> : (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.	54

Figure 4.23:	Closer view of section through ovary of <i>Cyperus esculentus</i> infected by <i>Claviceps cyperi</i> : (a) hyphal cells visible on the inside and (b) conidial layer covering ovary on the outside.	55
Figure 4.24:	Antagonism in culture between the <i>Sphacelia</i> state of <i>Claviceps cyperi</i> and <i>Fusarium heterosporum</i> .	56
Figure 4.25:	Antagonism in culture between the <i>Sphacelia</i> state of <i>Claviceps cyperi</i> and <i>Cladosporium cladosporioides</i> .	56
Figure 5.1:	Colony of <i>Claviceps cyperi</i> growing on Mantle's alkaloid medium.	68
Figure 5.2:	Lack of inhibition of <i>Fusarium heterosporum</i> by α -ergocryptine.	68
Figure 5.3:	Lack of inhibition of <i>Cladosporium cladosporioides</i> by α -ergocryptine.	68
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.	86
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.	87
Figure 6.3:	Dendrogram showing genetic differences between three <i>Claviceps</i> species and <i>Tilletia indica</i> based on multilocus fingerprinting data.	88
Figure 6.4:	Electrophoretic band patterns using BOXA1R primer.	89
Figure 6.5:	Electrophoretic band patterns using ARP-7 primer.	90
Figure 6.6:	Electrophoretic band patterns using ERIC-2 primer.	91
Figure 6.7:	Maximum parsimony tree based on multilocus fingerprinting profiles of three <i>Claviceps</i> species and <i>Tilletia indica</i> .	92
Figure 6.8:	Dendrogram showing genetic distances between different <i>Claviceps</i> species and related teleomorphic species based on ITS1/2 spacer sequences.	93
Figure 6.9:	Two-dimensional scatter plot showing genetic differences between <i>Claviceps</i> species and related teleomorphic species based on ITS 1/2 spacer sequence data.	94
Figure 6.10:	Phylogenetic tree showing phylogenetic relationships between <i>Claviceps</i> species and related teleomorphic species based on	

	ITS1/2 spacer sequence data.	95
Figure 6.11:	Dendrogram showing genetic distances between different <i>Claviceps</i> species and related teleomorphic species based on β -tubulin gene intron 3 region sequences.	96
Figure 6.12:	Two-dimensional scatterplot showing genetic distances between different <i>Claviceps</i> species and related teleomorphic species based on β -tubulin gene intron 3 region sequences.	97
Figure 6.13:	Phylogenetic tree showing phylogenetic relationships between <i>Claviceps</i> species and related teleomorphic species based on β -tubulin gene intron 3 region sequences.	98

LIST OF TABLES

Table 2.1:	Origin of <i>Claviceps cyperi</i> specimens from <i>Cyperus esculentus</i> included in the study.	21
Table 2.2:	Mean overall germination percentage of sclerotia of <i>Claviceps cyperi</i> .	22
Table 2.3:	Effect of temperature treatment on the germination of sclerotia of <i>Claviceps cyperi</i> .	22
Table 3.1:	Growth rate of the <i>Sphacelia</i> state of <i>Claviceps cyperi</i> on different media at different temperatures.	34
Table 5.1:	Ergopeptine alkaloid content of sclerotia of <i>Claviceps cyperi</i> collected between 1997 and 2003 from ergotised nut sedge implicated in bovine ergotism in South Africa.	69
Table 6.1:	Strains of <i>Claviceps</i> species and related teleomorphic genera included in this study.	99
Table 6.2:	Dendrogram and K-means clustering of South African isolates of <i>Claviceps purpurea</i> , <i>C. grohii</i> , <i>C. cyperi</i> and <i>Tilletia indica</i> based on multilocus fingerprinting.	101
Table 6.3:	Dendrogram and K-means clustering of different <i>Claviceps</i> species and related teleomorphic species showing genetic distances based on ITS 1/2 spacer sequence data.	102
Table 6.4:	Dendrogram and K-means clustering of different <i>Claviceps</i>	

species and related teleomorphic species showing genetic distances
based on β -tubulin gene intron 3 region sequence data.

103

CONTENTS

ACKNOWLEDGEMENTS	i
RESUMÉ	ii
LIST OF FIGURES	v
LIST OF TABLES	viii
CHAPTER 1 GENERAL INTRODUCTION	1
CHAPTER 2 SYMPTOMATOLOGY AND MORPHOLOGY OF <i>CLAVICEPS CYPERI</i> ON YELLOW NUT SEDGE IN SOUTH AFRICA	9
Abstract	9
2.1 INTRODUCTION	9
2.2 MATERIALS AND METHODS	10
2.3 RESULTS	11
2.4 DISCUSSION	13
2.5 REFERENCES	16
CHAPTER 3 THE <i>SPHACELIA</i> STATE OF <i>CLAVICEPS CYPERI</i> IN CULTURE	23
Abstract	23
3.1 INTRODUCTION	23
3.2 MATERIALS AND METHODS	24
3.3 RESULTS	27
3.4 DISCUSSION	28
3.5 REFERENCES	29

CHAPTER 4	MODE OF INFECTION OF <i>CYPERUS ESCULENTUS</i> BY <i>CLAVICEPS CYPERI</i>	35
	Abstract	35
4.1	INTRODUCTION	35
4.2	MATERIALS AND METHODS	36
	4.2.1 Microscopy	36
	4.2.2 Honeydew-colonising fungi	37
	4.2.3 Associated insects	37
4.3	RESULTS	37
	4.3.1 Microscopy	37
	4.3.2 Honeydew-colonising fungi	38
	4.3.3 Associated insects	38
4.4	DISCUSSION	39
4.5	REFERENCES	42
CHAPTER 5	ERGOT ALKALOIDS PRODUCED BY <i>CLAVICEPS CYPERI</i>	57
	Abstract	57
5.1	INTRODUCTION	57
5.2	MATERIALS AND METHODS	58
	5.2.1 Preparation of inoculum	58
	5.2.2 Culturing	59
	5.2.3 Extraction of alkaloids	60
	5.2.4 Alkaloid analysis	60
	5.2.5 Antimycotic activity of α -ergocryptine	61
5.3	RESULTS	61
5.4	DISCUSSION	62
5.5	REFERENCES	64

CHAPTER 6	MOLECULAR SYSTEMATICS OF <i>CLAVICEPS CYPERI</i> AND OTHER SOUTH AFRICAN <i>CLAVICEPS</i> SPECIES	70
	Abstract	70
6.1	INTRODUCTION	70
6.2	MATERIAL AND METHODS	71
	6.2.1 Strains examined	71
	6.2.2 Extraction and purification of DNA	72
	6.2.3 PCR fingerprinting of genomic DNA	72
	6.2.4 Sequence analysis of the complete ITS1/2 and 5.8S regions (rDNA operon)	73
	6.2.5 Amplification and sequencing of the β -tubulin gene intron 3 region	75
6.3	RESULTS	76
	6.3.1 Multilocus fingerprinting of genomic DNA	76
	6.3.2 Sequence analysis of ITS1/2 and 5.8S regions	77
	6.3.3 Sequence analysis of the β -tubulin gene intron 3 region	78
6.4	DISCUSSION	79
6.5	REFERENCES	82
CHAPTER 7	GENERAL DISCUSSION	104