SOME AEROBIC BLASTOCLADIOMYCOTA AND CHYTRIDIOMYCOTA CAN SURVIVE BUT CANNOT GROW UNDER ANAEROBIC CONDITIONS

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Abstract

In the present study twenty-two chytrids isolated aerobically from soils in Australia were tested for ability to grow or to survive under strict anaerobic conditions. These fungi were previously assigned to the orders Chytridiales, Blastocladiales, Rhizophydiales and Spizellomycetales by molecular techniques. None of the isolates grew in liquid growth media under strict anaerobic conditions. However, all twenty-two isolates survived in liquid growth media under strict anaerobic conditions for relatively short periods of time, which can occur periodically in the soil. Three of these isolates produced acid during growth in the presence of air indicating the capacity for lactic acid fermentation. Most members of the orders Chytridiales, Blastocladiales, Rhizophydiales and Spizellomycetales are considered to be obligate aerobes. Based on previous growth studies only two genera in these orders have been classified as facultative anaerobes. In contrast all members of the order Neocallimastigales are obligate anaerobes.

Key words: soil chytrids, Chytridiomycota, anaerobic growth, anaerobic survival, acid production.

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Introduction

Chytrids are true fungi that produce zoospores usually with a single posterior whiplash flagellum. These microorganisms are currently placed into two closely related phyla, the Blastocladiomycota and Chytridiomycota and six orders, the Blastocladiales, Chytridiales, Monoblepharidales, Neocallimastigales, Rhizophydiales and Spizellomycetales (James *et al.* 2000, Barr 2001, James *et al.* 2006, Letcher *et al.* 2006). Prior to the discovery of the Neocallimastigales, chytrids were thought to be primarily an obligately aerobic group of fungi (Gleason 1976, Barr 2001). Many chytrids in the orders Blastocladiales, Chytridiales, Monoblepharidales, Rhizophydiales and Spizellomycetales have been isolated from top soil and fresh water environments and a few from marine environments where the dissolved oxygen concentration is probably relatively high (Sparrow 1960, Karling 1977, Barr 1987, Whisler 1987, Barr 2001). In contrast the fungi in the Order Neocallimastigales have been isolated from the digestive systems of herbivorous vertebrates. All species in this order are considered to be obligate anaerobes (Orpin 1994, Trinci *et al.* 1994, Orpin & Joblin 1997, Rezaeian *et al.* 2004), that is, they cannot grow or survive in the presence of oxygen.

Nevertheless, fungi belonging to several genera in the Blastocladiales, Chytridiales and Monoblepharidales have been observed growing on substrates in stagnant water, mud and benthic sediments where the dissolved

oxygen concentration is probably relatively low (Sparrow 1960, Paterson 1967, Willoughby 1961, Karling 1977, Emerson & Natvig 1981, Whisler 1987, Dasgupta & John 1988, Steciow *et al*. 2001). For this reason some early researchers proposed that, based on observations in the field, at least a few of these fungi might be facultative anaerobes (Craseman 1954, Emerson & Robertson 1974, Emerson & Natvig 1981). Despite this, few chytrids growing in stagnant water, mud or benthic sediments have been isolated and grown in pure culture in the laboratory, and measurements of dissolved oxygen concentration were not available from the sites where they were observed. Their presence in stagnant water, mud and benthic sediments may be because these fungi can actually grow under anaerobic conditions or because they may just tolerate temporary anaerobiosis or reduced oxygen tensions. Furthermore, the capacity to grow or survive under anaerobic conditions may be more widespread among the Blastocladiales, Chytridiales, Monoblepharidales and Spizellomycetales than previously thought.

Isolates in only two genera of chytrids have been classified as facultative anaerobes in laboratory studies (Emerson & Cantino 1948, Cantino 1949, Craseman 1954, Craseman 1957, Emerson & Robertson 1974, Emerson & Natvig 1981, Lingle & Barstow 1983, Gleason & Gordon 1988, Gleason *et al*. 2002). These include four isolates from the genus *Blastocladia*, which grew under strict anaerobic conditions (Held *et al*. 1969, Gleason & Gordon 1988) and *Macrochytrium* (Craseman 1954), which grew under reduced oxygen tensions. In contrast, isolates in six other genera (*Allomyces*, *Catenaria*, *Chytridium*, *Cladochytrium*, *Rhizophlyctis* (*Karlingia*) and *Phlyctorhiza*) were unable to grow under anaerobic conditions or reduced oxygen tensions in the laboratory (Stanier 1942, Craseman 1954, Ingraham & Emerson 1954, Rothwell 1956, Goldstein 1960, Willoughby 1962, Nolan 1970). The methods used by Goldstein (1960), Rothwell (1956), Stanier (1942) and Willoughby (1962) did not adequately control the level of dissolved oxygen. Survival under strict anaerobic conditions has not been tested in any of the obligately aerobic chytrids.

Some members of the Chytridiomycota have the capacity for lactic acid fermentation in liquid media containing carbohydrates as substrates so that it is possible for them to produce ATP in the absence of oxygen. However the production of lactic acid during growth has been reported in only six genera in the Blastocladiales and Chytridiales: *Allomyces* (Ingraham & Emerson 1954, Turian 1960), *Blastocladia* (Emerson & Cantino 1948, Cantino 1949, Craseman 1957, Gleason & Gordon 1988), *Blastocladiella* (Cantino 1951, Cantino 1960), *Chytridium* (Craseman 1954), *Cladochytrium* (Willoughby 1962) and *Macrochytrium* (Craseman 1954). Nevertheless, lactic acid fermentation may also be possible in other genera.

Little is known about the ability of most soil chytrids to grow or survive under anaerobic conditions. The purpose of this research is to examine both growth and survival of soil chytrids under strict anaerobic conditions and to determine whether soil chytrids may continue to grow or survive in a dormant state without growth during periodic anaerobiosis.

Materials and Methods

Twenty-two isolates of chytrids listed in Table 1 were selected for the present study. The putative identity and origin of these isolates and some of the procedures used in the present study have been described previously (Gleason *et al.* 2004, Letcher *et al*. 2004a,b, Commandeur *et al.* 2005, Gleason *et al*. 2005, Gleason *et al*. 2006, Letcher *et al*. 2006). The fungi were isolated from natural and cropping soils collected near the surface and in contact with air at sites in New South Wales and Tasmania from three general types of habitat: (1) highly organic and/or clay, poorly aerated, high moisture content and poor drainage, (2) mixed organic and sandy, good aeration, often moist and good drainage and (3) mostly sandy, good aeration, usually dry and good drainage.

Preparation of inocula

All cultures were maintained on PYG agar medium (glucose 3.0 g L^{-1}, peptone 1.25 g L^{-1}, yeast extract 1.25 g L^{-1} and agar 20 g L^{-1}). The inocula for monocentric chytrids were prepared by flooding seven-day-old cultures growing on

Table 1. Survival of Chytrids isolated from soils in Australia after incubation under strict anaerobic conditions.

Fungus name	Order[1]	Survival[2] (days)		Fungus name	Order[1]	Survival[2] (days)	
		7	31			7	31
Allomyces arbuscula Allo Mar CW16	B	Y	Y	*Boothiomyces* sp. AUS 2	R	Y	Y
Catenaria anguillilae Poly Ad 2-0	B	Y	Y	*Terramyces* sp. AUS 3	R	Y	Y
Catenaria anguillilae Dec CC 4-10Z	B	Y	N	*Boothiomyces* sp. AUS 6	R	Y	Y
Spizellomyces sp. Mar Ad 2-0	S	Y	Y	*Boothiomyces* sp. AUS 7	R	Y	Y
Gaertneriomyces sp. Mar C/C2	S	Y	Y	*Boothiomyces* sp. AUS 8	R	Y	Y
Spizellomyces sp. Dec CC 4-10F	S	Y	N	*Boothiomyces* sp. AUS 9	R	Y	Y
Rhizophlyctis rosea AUS 13	S	Y	Y	*Boothiomyces* sp. AUS 12	R	Y	N
Powellomyces sp. AUS 16	S	Y	N	*Kappamyces laurelensis* AUS 15	R	Y	Y
Powellomyces sp. AUS 17	S	Y	N	*Cladochytrium* sp. AUS 11	C	Y	Y
Rhizophydium sp. Mar Ad 14	R	Y	Y	*Chytriomyces hyalinus* AUS 14	C	Y	Y
Rhizophydium sp. Mar R2	R	Y	N	*Chytriomyces hyalinus* Ob 3-8	C	Y	Y

[1] B: Blastocladiales, C: Chytridiales, R: Rhizophydiales, S: Spizellomycetales. [2] Y: Yes, N: No.

PYG agar in 25 mL Petri dishes with 5 mL of de-ionized water. After two hours the resulting zoospores and sporangia were mixed with a transfer loop. Approximately 0.5 mL of a mixture of zoospores and sporangia was inoculated into the sterile media in the serum bottles using a sterile syringe with an 18-gauge needle. The inocula for polycentric and hyphal forms were prepared by grinding cells grown in liquid or solid PYG media with a micropestle in a 1.5 mL microcentrifuge tube. 0.5 mL of the resulting slurry was inoculated into sterile media.

When inocula with only zoospores and recently encysted zoospores were needed, seven day old lawns instead of clumps of thalli were first prepared on the surface of the solid PYG media. The lawn was then flooded with 5 mL of de-ionized water. The thalli remained attached to the surface of the solid medium, so that the inocula consisted of only zoospores and recently encysted zoospores in de-ionized water. All inocula were examined for composition with the light microscope.

Growth in anaerobic media

The general procedures for testing for growth of fungi under strict anaerobic conditions have been described previously by Gleason and Gordon (1988, 1989) but were modified slightly for the present study. The liquid growth media contained glucose 3.0 g L^{-1}, peptone 1.25 g L^{-1}, yeast extract 1.25 g L^{-1}, K_2HPO_4 1.74 g L^{-1} (buffer), $MgSO_4$ 0.12 g L^{-1}, cysteine. HCl 1.00, 0.3 or 0.0 g L^{-1} and either resazurin for oxygen detection or brom cresol

Table 2. Complex liquid media used to test for growth under aerobic, reduced oxygen tension and strict anaerobic conditions.

Growth medium number	Environment	Cysteine (g L^{-1})	Resazurin	Brom cresol purple	Bubbled with oxygen-free nitrogen
1	Anaerobic	1.0	+	-	+
2	Anaerobic	0.3	+	-	+
3	Reduced oxygen	0	-	+	+
4	Aerobic	0	-	+	-

In anaerobic growth media 1 and 2 the redox potential was held below -100 mV with the addition of cysteine, a reducing agent. The oxygen tension of growth medium 3 was reduced below 0.1% by bubbling with high purity oxygen-free nitrogen. The oxygen tension of the aerobic growth medium 4 was not changed during preparation.

purple for acid detection at 1 mg L^{-1}. Fifty mL of media was dispensed into each 100 mL serum bottle (Alltech). The media were then bubbled with high purity nitrogen for at least one minute. The bottles were closed with butyl rubber stoppers and aluminium crimp seals without allowing air to enter. Bottles were sterilized at 121°C for 15 minutes.

The four liquid growth media listed in Table 2 were prepared to test for aerobic and anaerobic growth. The incubation time for the experiments with media 1, 2 and 3 was 22 days. The growth experiment with medium 4 was stopped at seven days due to the rapid growth of all chytrids except for *Catenaria* sp. Poly Ad 2-0 and *Cladochytrium* sp. AUS 11. The latter two cultures were incubated for 22 days because of slow growth. In addition, as a control for each experiment, the inoculum for each isolate was placed onto the surface of 25 mL of solid PYG medium with 2% agar in Petri dishes (medium 5) and incubated for seven days to test for viability. At first all growth experiments included only one replicate of each isolate, and then the growth experiments with medium 1 and medium 5 (control) were repeated twice with each isolate for 22 days. Growth experiments with *Allomyces arbuscula* Allo Mar CW 16, *Catenaria* sp. Poly Ad 2-0 and *Cladochytrium* sp. AUS 11 in medium 4 were repeated three times.

Growth was monitored visually for up to 22 days at 20°C. The appearance of new clumps or increase in volume of clumps of thalli was taken to indicate growth. None of the cultures were shaken. The pH of the medium was monitored by observing the color of brom cresol purple during growth in experiments with media 3 and 4 by the method of Emerson (1958). Brom cresol purple changes color from purple to yellow when the pH drops below pH 6.0. In addition the pH was measured with a pH meter at harvest in medium 4. The redox potential was monitored by observing the color of the redox indicator resazurin ($E0^1$= -42 mV) (Gleason & Gordon 1988). Resazurin changes color from colorless to blue when the culture medium is oxygenated.

Survival in anaerobic medium

In two separate experiments, 0.5 to 1.0 mL samples of a mixture of zoospores, zoospore cysts and thalli in de-ionized water were inoculated into the liquid growth medium 1 in serum bottles and onto the solid medium (medium 5) in Petri dishes (for the control) and incubated for 7 and for 31 days. Then the contents of the serum bottles were poured into 50 mL centrifuge tubes. Following centrifugation most of the liquid medium was removed from the cultures. The thalli with some of the remaining liquid medium were transferred onto solid PYG agar in Petri dishes (recovery medium, medium 5) and examined for resumption of growth during aerobic incubation for seven days at 20°C.

In a third experiment serum bottles containing the liquid growth medium 1 were inoculated with 1 mL of de-ionized water containing only zoospores and recently encysted zoospores of AUS 6, AUS 14, Mar Ad 14 and Mar Ad 2-0. These cultures were incubated for one or for seven days. The contents of each serum bottles were poured into two 50 mL sterile centrifuge tubes. The second 25 mL of growth media was split between three Petri dishes. A one mL sample from the bottom of each Petri dish was spread onto the surface of solid PYG

medium in a Petri dish (recovery medium). The cultures were examined for resumption of growth during aerobic incubation for seven days at 20°C. The number of colonies on the surface of the recovery medium in each Petri dish was recorded. Each colony was examined with the dissecting microscope to verify that it had developed from a single zoospore or zoospore cyst.

Results

Growth under aerobic, reduced oxygen and anaerobic conditions

None of the twenty-two fungi (Table 1) grew during incubation for 22 days in anaerobic liquid media 1, 2 and 3. All of the fungi grew well during incubation for 7 days in aerobic liquid medium (medium 4) and on the aerobic solid medium (medium 5, the control).

The color of brom cresol purple in medium 3 (anaerobic) remained unchanged during the 22-day incubation period in all isolates. In medium 4 (aerobic) the color of brom cresol purple did not change during the growth of all of the isolates during the 7-day incubation period except for *Allomyces arbuscula* Allo Mar CW 16, where the color of the medium changed from purple to bright yellow before the end of the 7-day incubation period. In separate experiments with medium 4 (aerobic) the slower growing isolates of *Cladochytrium* sp. AUS 11 and *Catenaria* sp. Poly Ad 2-0 caused the color of brom cresol purple to change from purple to bright yellow during growth between 8 and 22 days.

In the initial experiment with medium 4 the pH was measured after the 7 days with a pH meter and remained above 6.0 with all isolates except for *Allomyces arbuscula* Allo Mar CW 16. In separate experiments with medium 4 the pH of three cultures of *Allomyces arbuscula* Allo Mar CW 16, *Cladochytrium* sp. AUS 11 and *Catenaria* sp. Poly Ad 2-0 was measured with a pH meter at the end of the 7, 14 and/or 22 days. The mean pH values (± 1 standard deviation) for Allo Mar CW 16 were pH 5.9 ± 0.1 at 7 days and 4.8 ± 0.01 at 14 days. The mean pH values for AUS 11 were pH 5.7 ± 0.1 at 14 days and 4.9 ± 0.2 at 22 days. The mean pH values for Poly Ad 2-0 were pH 5.7 ± 0.1 at 14 days and 5.2 ± 0.03 at 22 days.

Survival under anaerobic conditions

All of the fungi resumed rapid growth immediately after being transferred to the recovery medium following incubation under anaerobic conditions for 7 days (Table 1). Sixteen of the fungi (*Allomyces arbuscula* Allo Mar CW 16, *Catenaria anguillilae* Poly Ad 2-0, *Spizellomyces* sp. Mar Ad 2-0, *Gaertneriomyces* sp. Mar CC2, *Rhizophlyctis rosea* AUS 13, *Chytriomyces hyalinus* AUS 14 and Ob 3-8, *Cladochytrium* sp. AUS 11, *Boothiomyces* sp. AUS 2, AUS 6, AUS 7, AUS 8, AUS 9, *Terramyces* sp. AUS 3, *Kappamyces laurelensis* AUS 15 and *Rhizophydium* sp. Mar Ad 14) also resumed growth following incubation under anaerobic conditions for 31 days (Table 1).

In experiments using only zoospores and zoospore cysts as the inoculum, AUS 6, AUS 14, Mar Ad 14 and Mar Ad 2-0 were incubated for one or for seven days under anaerobic conditions and then transferred to recovery medium. In two separate experiments no colonies were obtained for Mar Ad 2-0 after anaerobic incubation for one and for seven days. With the other fungi after incubation for seven days under aerobic conditions the mean number of colonies (± 1 standard deviation) per plate was 13 ± 3 for AUS 6, 20 ± 1 for AUS 14, and 0 for Mar Ad 14.

Discussion

None of the isolates in the present study grew under strict anaerobic conditions in media 1 and 2 or under reduced oxygen tensions in medium 3 during the 22-day incubation period. Thus the growth of these soil chytrids appears to be very sensitive to low dissolved oxygen concentrations. The results reported in the present study with medium 3 are consistent with data previously reported for other isolates in several genera of chytrids (Stanier 1942; Craseman 1954; Ingraham & Emerson 1954; Rothwell 1956; Goldstein 1960; Willoughby 1962; Nolan 1970). Furthermore, the data support the hypothesis that most members of the Chytridiales, Blastocladiales, Rhizophydiales and Spizellomycetales are obligate aerobes.

Survival under strict anaerobic conditions had not been tested previously in any of the obligately aerobic chytrids. However all of the

isolates in the present study survived incubation for seven days and most isolates for 31 days under strict anaerobic conditions. Thus these fungi appear to tolerate periodic anaerobiosis, enabling continuation in the soil through periods of flooding or similar perturbations.

Although we could not observe growth visually under anaerobic conditions during the 21-day incubation time, it is possible that extremely slow growth may have occurred in some isolates. However, it is more likely that all of these chytrids became dormant at the onset of anaerobiosis and resumed growth later after the re-introduction of oxygen. The capacity of these chytrids to survive anaerobiosis beyond 31 days is unknown.

The stage in the life cycle that is resistant to anaerobic conditions is not known. Zoospores of all fungal structures are probably the least tolerant of stressful conditions. Zoospores and/or recently encysted zoospores of *Boothiomyces* sp. AUS 6 and *Chytriomyces hyalinus* AUS 14 but not *Rhizophydium* sp. Mar Ad 14 or *Spizellomyces* sp. Mar Ad 2-0 survived anaerobic conditions for seven days. The mature thallus however appears likely to be the structure that is most resistant to the lack of oxygen.

The method used in the present study to insure the maintenance of strict anaerobic conditions (with a redox potential below -100 mV) appeared to be satisfactory. Even with the introduction of a small amount of oxygen in the inoculum, the resezurin did not change color from colorless to blue during incubation.

None of the isolates in the present study were strongly fermentative and facultatively anaerobic like *Blastocladia* (Gleason & Gordon, 1988). Three isolates in the present study, *Allomyces* Allo Mar CW 16, *Cladochytrium* AUS 11 and *Catenaria* sp. Poly Ad 2-0 released enough acid into the growth medium with 10 mM phosphate buffer to lower the pH below 6. The fact that fungi in two of these genera are strong acid producers has been reported previously by Ingraham & Emerson (1954), Turian (1960) and Willoughby (1962). This indicates that at least three of the isolates used in the present study can ferment glucose to lactic acid, which is a process that does not require oxygen.

The reasons for the inability of soil chytrids to grow under low oxygen tensions or strict anaerobic conditions are not known. It is possible that these fungi cannot synthesize essential molecules in the absence of oxygen, that essential nutrients are not provided by peptone and yeast extract in the medium and/or that these fungi lack the metabolic machinery to produce enough ATP by fermentation for biosynthesis. In contrast, rumen fungi grow well in synthetic media under strict anaerobic conditions (Orpin 1994). It is interesting to note that the cells of one isolate of *Allomyces* pre-grown aerobically can continue to ferment carbohydrates but cannot grow under anaerobic conditions (Ingraham & Emerson 1954). This suggests that it is possible for some chytrids to produce enough ATP by fermentation to insure survival during short periods of anaerobiosis.

The purpose of the present study was to test chytrids isolated from aerated soils for the ability to grow and survive under anaerobic conditions. Because only a small number of isolates were tested, it cannot be concluded that facultatively anaerobic chytrids do not occur in aerated soils. Furthermore, it is possible that if the isolation procedures were carried out under strict anaerobic conditions, facultatively and obligately anaerobic chytrids could be found in samples of compacted soil, stagnant water, mud and benthic sediments. Special procedures and equipment may need to be adapted for isolations of potentially anaerobic chytrids, such as enrichment with reduced dissolved oxygen concentrations (Whisler 1987) and anaerobic growth chambers (Theodorou *et al.* 1994, Trinci *et al.* 1994, Orpin & Joblin 1997, Rezaeian *et al.* 2004).

The chytrids used in the present study were isolated from various soil types and climates in Australia. All twenty-two chytrids appeared to survive but could not grow during short periods of anaerobiosis. The capacity to tolerate anaerobiosis appears to be similar in chytrids isolated from clay and sand textured soils and hot and cold climates. While mature sporangia are the most likely survival

structure, some chytrids may also survive as zoospores or zoospore cysts. Without growth it is possible that only small quantities of nutrients are needed for survival through these periods of anaerobiosis. The mechanisms underlying responses to anaerobiosis in soil chytrids remain to be elucidated.

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THE FUNGUS *LECANICILLIUM LECANII* COLONISES THE PLANT *GOSSYPIUM HIRSUTUM* AND THE APHID *APHIS GOSSYPII*

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Abstract

The purpose of the research reported here was to determine whether the fungus *L. lecanii*, which was isolated as an endophyte from cotton (*Gossypium hirsutum*), may readily colonise cotton, an aphid pest of cotton (*Aphis gossypii*), and transfer from plant to aphid, and from aphid to plant.

L. lecanii from agar culture and growing on or in alternative hosts was used to inoculate the aphid *A. gossypii* and cotton *G. hirsutum.* Colonisation was assessed by isolating the fungus from the surface or from within each host. *L. lecanii* colonised each host from spores, and transferred from aphid to cotton and cotton to aphid. The fungus sporulated on the surface of both hosts. Internal colonisation of each host increased over time under certain conditions.

In conclusion, the entomopathogen *L. lecanii* readily colonised two potential hosts and transferred between the hosts under experimental conditions. *L. lecanii* is widely used in glasshouses to control aphids. The potential for the entomopathogen to survive in field conditions is indicated by these results. If the outcome is supported under field conditions, the fungus may be used to reduce the impact of a pest of cotton in Australia.

Key words: *Verticillium lecanii,* endophyte, epiphyte, entomopathogen, tripartite interaction, biocontrol.

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Introduction

A wide variety of fungi, referred to as endophytes, are asymptomatic colonists of plants. The relationship between endophyte and plant is understood in a few cases. For instance, seed-transmitted *Neotyphodium* spp. colonise grasses, modifying interactions between plant and insects. Laterally transmitted endophytes appear less specific in association, and they must grow in the presence of hostile plant metabolites (Schultz *et al.* 1999).

The fungus *Lecanicillium lecanii* (Zimmerman) Zare & Gams was isolated from within asymptomatic leaves of cotton *Gossypium hirsutum* L. taken from an irrigated field near Narrabri, Australia (McGee 2002). Some isolates of this fungus are mycoparasites (Kim *et al.* 2007). However, the absence of other fungi at isolation would suggest that our

isolate was not associated with another fungus. *L. lecanii* is also an entomopathogen used to control aphids and whiteflies in glasshouses (Inglis *et al.* 2001) where high humidity prevails (Gillespie & Claydon 1989). The lifecycle of *L. lecanii* is poorly documented.

Endophytic colonisation of maize (Bing & Lewis 1992) and epiphytic occupation of leaf surfaces (Meyling & Eilenberg 2006) by the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin indicates that some entomopathogens may persist on plants in the absence of insect hosts. The plant provides the fungus with a nutritious, stable and long lived (relative to an insect) environment, with the added advantage that contact with an insect host is possible. Thus *L. lecanii* may have two hosts: insect and plant. Alternatively, the isolate of *L. lecanii* from cotton may be an endophyte lacking the capacity to colonise an insect host.

The potential for transfer by *L. lecanii* between different hosts is unknown. If transfer takes place, then the tripartite interaction may have potential to regulate damaging populations of insect pests in the field. Interactions between three different organisms are simpler to study in controlled conditions than the field. The purpose of the research reported here was to determine whether *L. lecanii* may readily colonise cotton *G. hirsutum*, an aphid pest of cotton *Aphis gossypii* Glover, and transfer from plant to aphid, and from aphid to plant.

Materials and methods

A colony of *A. gossypii* was established on cotton seedlings (cv. Sicala V2) growing in sterilised potting medium (McGee *et al.* 1997) and maintained in a growth cabinet with a 13/11 h day/night cycle (28/22 C). Photosynthetically active radiation was approximately 350 µmol m^{-2} s^{-1} at the soil surface.

L. lecanii from cotton (McGee 2002) was cultured on potato dextrose agar (PDA) at 23°C. A spore/mycelium suspension (approx. 1 × 10^7 spores mL^{-1}) was harvested from cultures in sterile de-ionised water and used to inoculate aphids and leaves of cotton (Kanagaratnam *et al.* 1982).

The potential for *L. lecanii* to colonise *A. gossypii*

A surface sterilised leaf of cotton (cv. Sicala V2), from a plant maintained in an aphid-free growth cabinet, was placed on a disk of moistened sterile filter paper in a Petri dish (15 cm diameter). Twenty medium-sized adult apterous aphids were placed on the leaf. Pilot studies indicated that aphids readily fed on excised leaves for up to 14 d. Aphids were sprayed either with a spore suspension of *L. lecanii* or sterile de-ionised water as the control. Petri dishes were sealed with Parafilm™. After 7 d, mortality among aphids was calculated as the number of dead aphids in each of five replicate Petri dishes expressed as a percentage of the total number of live and dead aphids in the dish. A sub-sample of live aphids from inoculated and uninoculated dishes was either surface sterilised by dipping briefly in ethanol (70% w/v), or taken directly from a dish, and pressed gently into PDA. The aphids were incubated at 23°C for 7 d and emergence of *L. lecanii* noted. The experiment was repeated. Pooled *t*-tests were used to detect differences in mortality.

The potential for *L. lecanii* to colonise cotton

Treatments

Cotton leaves may be colonised by airborne spores of the fungus. Alternatively, the fungus may be vectored by aphids. Growth and subsequent colonisation of cotton by the fungus may be enhanced by the presence of aphid exoskeletons and honey dew, which are both common on aphid infested plants. Endophytic colonisation of cotton may be enhanced by the disruption of the epidermis that occurs when aphids feed (Pollard 1973). Therefore we designed treatments to mimic conditions at the leaf surface that may occur during aphid infestation. Sucrose (2% w/v) was added to the inoculum to provide additional energy to fungal spores for colonisation of leaves as would be present on leaves saturated with aphid honey dew. Chitin (0.025 g mL^{-1}) was added to inoculum to simulate shed aphid exoskeletons. Fine emery paper was gently pressed once against the adaxial leaf surface to cause minor disruption of the epidermis and allow direct penetration of fungal spores into the leaf. This "leaf

damage" treatment was not a direct simulation of aphid damage, as this is not possible. It was intended to allow easier access of fungal spores into the leaf during inoculation, as is expected to occur on leaves damaged by aphid feeding.

Inoculum of *L. lecanii* with the surfactant Pulse™ at a rate of 0.02% w/v was applied to the adaxial leaf surface with a camel hair brush. Seven treatment combinations were chosen: 1) spores in sucrose; 2) spores mixed with chitin; 3) spores in water applied to a damaged leaf; 4) spores mixed with chitin applied to a damaged leaf; 5) spores in sucrose applied to a damaged leaf; 6) spores in sucrose and chitin with no leaf damage; and 7) spores in sucrose and chitin with leaf damage. We controlled these treatments by applying spores in water with Pulse™ to undamaged leaves. Leaves were wrapped with clear plastic food wrap following inoculation to retain high relative humidity at the leaf surface. The plastic wrap was removed after 2 d.

Each treatment was randomly applied to a half-leaf (as delineated by the mid-vein) of the four youngest fully expanded leaves on a plant at the six true leaf stage. Each half leaf was considered to be one replicate. Five groups of five plants were inoculated, so there were five replicates (half leaves) per treatment in each group of five plants. The five groups would then be randomly selected at different points in time for re-isolation of the fungus.

Isolation

Five plants were randomly chosen and leaves removed for isolation of *L. lecanii* at seven, 14, 28, or 35 d after inoculation. Each half-leaf was pressed against PDA to determine whether the fungus was present on the leaf surface. Leaves were then surface-sterilised and incubated on PDA to determine whether the fungus was present inside the leaf. A sub-sample of surface sterilised leaf fragments was pressed against PDA to determine the effectiveness of surface sterilization (Dingle & McGee 2003). Plates were kept at 23°C for 7 d and then examined microscopically for the emergence of *L. lecanii*. The frequency of isolation of *L. lecanii* from the surface and within leaves was compared using likelihood Chi-square analysis to determine dependency between isolation and inoculation treatment. A sub-sample of inoculated leaves was also examined microscopically to determine where spores were located, whether or not spores had germinated and grown into the leaf, and where the fungus penetrated the leaf surface.

Transfer of *L. lecanii* from inoculated aphids to uninoculated cotton

Five leaves, heavily infested with *A. gossypii*, were each placed on disks of moistened filter paper in Petri dishes. Aphids were sprayed with a suspension of *L. lecanii* and dishes were sealed with Parafilm™. After 24 h, 15 aphids from each dish were carefully transferred with a camel hair brush onto each of five fresh uninoculated cotton leaves under the same experimental conditions. The brush was briefly soaked in 70% ETOH to kill any spores on the brush. After 6 d, aphids were removed from leaves, leaves were cut into fragments, pressed against PDA to determine colonisation of the leaf surface, and then surface-sterilized in 70% ETOH and 5% hypochlorite for 5 minutes and placed on PDA to determine internal colonisation by *L. lecanii*. A sub-sample of aphids was also pressed into PDA, or surface sterilised by briefly soaking in 70% ETOH and then pressed into PDA, incubated for 7 d, and examined for emergence of *L. lecanii*.

Transfer of *L. lecanii* from inoculated cotton to uninoculated aphids

Leaves of similar size and age on cotton plants were either 1) inoculated with *L. lecanii*, or 2) painted with sterile distilled water, or 3) left untreated. Experimental leaves were excised from the plant 7 d after inoculation, rinsed in sterile distilled water, and placed adaxial side down on a moistened 5 cm disk of Whatman no. 1 filter paper in a Petri dish (15 cm diameter). Ten uninoculated aphids were placed on each leaf. Dishes were sealed with Parafilm. After 7 d, a sub-sample of aphids from each treatment was placed on PDA, or surface sterilised and then placed on PDA. A sub-sample of leaf fragments from each treatment was also pressed against PDA, and then surface-sterilized and placed on PDA. Aphids, leaf presses and leaf fragments were incubated for one week and then examined for emergence of *L. lecanii*.

Results

The potential for *L. lecanii* to colonise *A. gossypii*

The mortality of inoculated aphids was 50 and 56% in each experiment. The mortality of uninoculated aphids was 15 and 10% in each experiment respectively, and these differences were significant in each experiment ($P < 0.01$). *L. lecanii* was isolated from two of 15 and 6 of 25 inoculated aphids previously surface sterilized with ethanol, and from 14 of 20 and 15 of 15 un-sterilized aphids, respectively. The fungus was not isolated from any uninoculated aphids. The fungus was also observed growing from the exoskeleton of living aphids and was apparent on shed exoskeletons and cadavers. Some colonised, dead aphids were attached by hyphae to the inner surface of the Petri dish.

The potential for *L. lecanii* to colonise cotton

Endophytic colonisation

Isolation of *L. lecanii* from surface sterilised cotton leaves was dependent upon the method used to apply inoculum (Likelihood $\chi^2 = 68.62$, $p < 0.001$, df = 7) and the length of time from inoculation to isolation (Likelihood $\chi^2 = 30.09$, $p < 0.001$, df = 4). *L. lecanii* was isolated more frequently from damaged surface sterilised leaves ($y = 103.875x - 0.75$, $r^2 = 0.316$, $n = 16$, $P = 0.233$) than undamaged surface sterilised leaves ($y = 53.813x - 1.06$, $r^2 = 0.574$, $n = 16$, $P = 0.02$) over time. The fungus was isolated from 100% of damaged leaves after 35 d but much less frequently from undamaged leaves.

Epiphytic colonisation

Inoculation treatment did not influence isolation of *L. lecanii* from the surface of leaves (Likelihood $\chi^2 = 11.82$, $p = 0.107$, df = 7). Frequency of isolation of the fungus from the leaf surface decreased as time from inoculation increased (Likelihood $\chi^2 = 45.16$, $p < 0.001$, df = 4). The decrease was linear over time ($y = 114x - 2.068$, $r^2 = 0.63$, $n = 32$, $P < 0.001$).

Microscopy

Spores of *L. lecanii* were aggregated adjacent to veins of inoculated leaves. Spores germinated on the leaf epidermis and appeared to penetrate directly from appressoria on the surface of epidermal cells and between epidermal cells.

The potential for *L. lecanii* to transfer from inoculated aphids to uninoculated cotton

L. lecanii was isolated from the surface of 100% of leaf fragments, and from within an average of 35% of leaf fragments after 7 d. The fungus was also isolated from 82% of non surface-sterilised aphids, but not from surface sterilised aphids.

Transfer of *L. lecanii* from inoculated cotton to uninoculated aphids

L. lecanii was isolated from 75% of aphids on inoculated leaves and from no aphids from uninoculated leaves. The fungus was not isolated from surface-sterilised aphids. The fungus was present at the surface of all inoculated leaves, and was detected on one uninoculated leaf, indicating minor cross-contamination potentially during the isolation process.

Discussion

An apparently endophytic isolate of *L. lecanii* colonised leaves of cotton and aphids under the experimental conditions. The fungus transferred from leaf to insect and insect to leaf. Though the degree of colonisation differed between experiments, the fungus killed aphids, and did not cause obvious disease in leaves of cotton. While the fungus did not always kill aphids within 7 d, in two experiments direct inoculation with spores resulted in substantial mortality, indicating that density of inoculum and time are directly related to aphid mortality. While the fungus did not persist for long on the leaf surface, it was isolated from within leaves up to 35 d after inoculation suggesting an endophytic life strategy. Longer incubation and leaf damage increased the likelihood of the inoculated fungus being recovered from within the leaf suggesting that insects are important in the establishment of this isolate of *L. lecanii* as an

endophyte in cotton. Microscopic examination confirmed the presence of the fungus on the surface of leaves and indicated that spores of the fungus germinated and grew on the leaf surface, and penetrated the leaf following inoculation. While leaf damage increased colonisation, germ tubes of the fungus directly penetrated surfaces of leaves, indicating possible fungal colonisation of intact leaves. *L. lecanii* has the potential to colonise both the leaves of cotton and one aphid pest of cotton, confirming the status of the isolate of *L. lecanii* used in this study as both endophyte and entomopathogen.

The high humidity under our experimental conditions was intended to enhance colonisation of plant and insect, and to investigate the possibility of transfer between hosts. Colonisation of insects by *L. lecanii* is increased under conditions of high humidity (Gillespie & Claydon 1989). Utilization of *L. lecanii* as a biocontrol agent of *A. gossypii* in the field will require periods of high humidity to facilitate colonisation of insect and plant. Extended periods of high humidity do occur in cotton fields in Australia, specifically during the middle of the growing season, when the canopy of the crop is closed and the crop is irrigated weekly (pers. comm. Dr L.J. Wilson CSIRO Entomology, Narrabri, Australia). In addition, *A. gossypii* is most commonly found in the lower canopy (Hardee *et al.* 1994), where humidity is high, thus indicating potential for direct contact between the fungus and aphid within the closed canopy.

Conidia of *Lecanicillium* spp. are unlikely to survive exposure to environmentally realistic levels of ultraviolet (UV) radiation (Braga *et al.* 2002). Sadras & Wilson (1997) demonstrated that the canopy of a mid-late season cotton crop can intercept > 80% of photosynthetically active radiation. Consequently, it is unlikely that conidia in the lower canopy are exposed to deleterious levels of UV radiation. However, this remains to be tested.

These findings indicate potential for an interaction between *L. lecanii*, *A. gossypii* and cotton in the field that may explain the isolation of endophytic *L. lecanii* from field grown cotton. Colonisation of the plant provides the fungus with a stable and nutritious insect-attracting environment. If the aphid reached pest densities, leaf damage may increase the likelihood of fungal colonisation of plant tissues. In addition, the presence of endophytic colonies might increase colonisation of aphids. Colonised aphids may disperse the fungus between plant tissues, and plants, becoming foci of inoculum *post mortem*. Lastly, the fungus may also parasitise fungi associated with plants and aphids. These hypotheses remain to be explored under field conditions.

The presence of the fungus within the plant may induce plant responses (Schultz *et al.* 1999) resulting in changes in aphid behavior (Underwood 1999). Thus good reason exists to explore the potential for this fungus to reduce feeding and/or reproduction of aphids in the field.

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A NEW GREEN SPECIES OF *HUMIDICUTIS* FROM WESTERN AUSTRALIA

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Abstract

A new species of the Hygrophoraceae, *Humidicutis viridimagentea*, is described from Western Australia and is also the first record of the genus from that state.

Key words: new species, *Humidicutis viridimagentea*, Hygrophoraceae, systematics, Australia.

A.M. Young and K. Syme (2007). A new green species of *Humidicutis* from Western Australia. *Australasian Mycologist* 26 (2-3): 71-74.

Introduction

Although eight species of *Humidicutis* (Singer) Singer have been recorded for Australia, all of these are so far known only from the eastern section of the continent (Young 2005). *Humidicutis viridimagentea* is therefore the first representative of the genus recorded for Western Australia. Apart from the definitive criterion of the genus (the absence of clamp connections throughout the basidioma except for the basidial bases), the new taxon exhibits other common characteristics of the genus such as the radial splitting of the subumbonate pileus, the basidial clamps of medallion to toroidal form and the regular pileal and lamellar tramas with chains of fusiform, inflated elements. Its green colouration also occurs in other Australian and New Zealand taxa within the genus.

Investigation of the eastern Australian Hygrophoraceae remains unfinished, but the number of known species has increased (over an 11 year period of investigation) from approximately 25 to 92 taxa and there are firm indications that many more new species still remain to be described for Australia as a whole (Young 2005). Given the comparatively unknown status of the Western Australian component of the Hygrophoraceae, it is very likely that more species within this genus will be found.

Materials and Methods

Two herbarium collections from PERTH and MEL form the basis of this study. Specimen samples were examined under an Olympus CX40 research light microscope (with drawing tube) using ammoniated Congo red as the mountant. Colour codes cited in the description are referenced to Kornerup & Wanscher (1981).

Humidicutis viridimagentea A.M. Young & K. Syme, *sp. nov.* (Figs 1, 2 & 3 A,B)

Etymology: indicating the principal colours; *viridis* (Lat.) - green; *magentea* (Lat.) - magenta.

Pileus (12-)24-36(-58) mm, atroviridis, conicus tum subumbonatus diende latus, glaber, lubricus vel siccus, ad marginem concolorum fissum. Lamellae sinuatae vel adnatae, flavae, ad marginem concolores. Stipes (35-)43-85 × (2.5-)5-8(-18) mm, viridis, siccus, glaber, cylindricus, cavus. Basidiosporae 6.5-8.0 × 4.5-6.0 µm, lato-ellipsoideae, hyalinae, numquam constrictae. Basidia 37-45.5 × 7-8.5 µm, 4-spora, fibulata. Cystidia nulla. Trama hymenophoralis regularis, sine fibulata.

Figure 1. *Humidicutis viridimagentea* amongst moss and litter. The deep bottle green colour of the pileus is visible in the two specimens at the very centre of the image. © R. Robinson

Epicutis pilei ixocutem eformans. Gregaria vel caespitosa in musca.

Holotypus hic designatus: Western Australia. Denmark, 34°58′44″S 117°13′52″E, 8.vii.2001, *K. Syme 1148/01*, (*holotypus* PERTH 07477635; isotypes BRI AQ742004 and MEL 2300381).

Pileus (12-)24-36(-58) mm, at first deep green (27F5) drying to a lighter green (26E5) then developing areas of magenta colouration (12B6) which may be darker (11E6) at the centre, conical becoming broadly conical then near campanulate or subumbonate and finally plane, smooth becoming rimose, lightly lubricous but then rapidly dry, hygrophanous; margin concolorous, at first incurved, crenulate, often splitting. *Pileal trama* thin, concolourous with the pileal surface when moist but becoming white when dry. *Lamellae* sinuate or adnate with a small decurrent tooth, deep yellow (3A7-3B7), at times with greenish tints near pileal undersurface, subdistant, sometimes forking, occasionally with veins on the lamellar faces; margins concolourous and even. *Stipe* (35-)43-85 × (2.5-)5-8(-18) mm, green (27D5-26D4) near the lamellae and extending downwards to about two thirds of the stipe length then becoming white towards the base, magenta tints appear in the green area with age and the white base may also become pinkish, smooth or sometimes with slight horizontal ridges, dry, hollow, cylindrical and may be either tapered or inflated towards the base. *Spore print* white.

Basidiospores 6.5-8.0 × 4.5-6.0 µm, mean 7.2 × 4.9 µm, Q: 1.3-1.6, mean Q: 1.47, broadly ellipsoid, hyaline, smooth, often with large inclusion, constrictions absent. *Basidia* 37-45.5 × 7-8.5 µm, mean 40.3 × 7.8 µm, Q: 4.6-5.9, mean Q: 5.15, 4-spored, clamp connections present of medallion form and frequently toroidal. *Cystidia* absent. *Hymenophoral trama* regular, composed of thin-walled, hyaline, cylindrical to fusiform, inflated, septate hyphal elements 40-130 × 5-25 µm, clamp connections absent. *Pileipellis* a weak ixocutis composed of thin-walled, hyaline, cylindrical, septate hyphae 2-4 µm diam., clamp connections absent. *Stipitipellis* a cutis composed of thin-walled, hyaline, cylindrical,

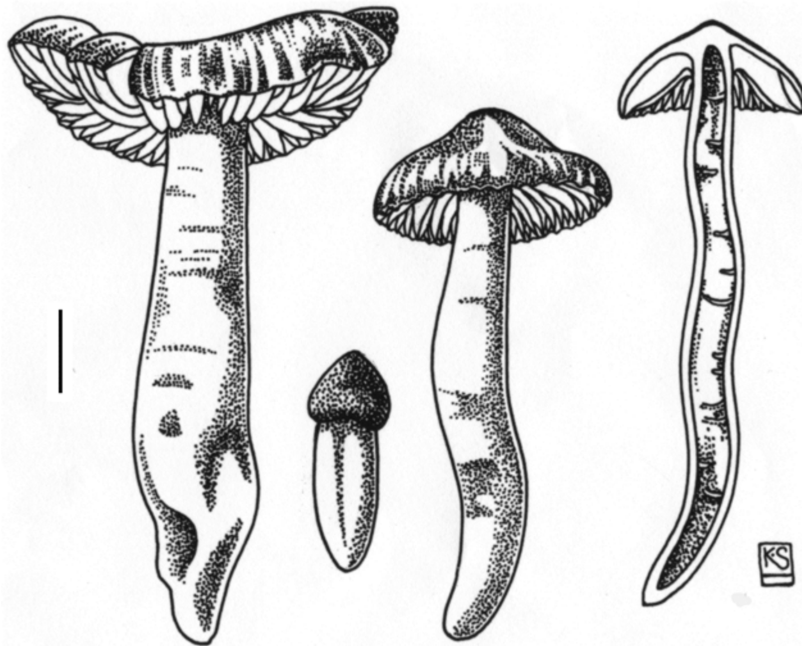

Figure 2. *Humidicutis viridimagentea* habit sketch. Scale bar = 1 cm. © K. Syme

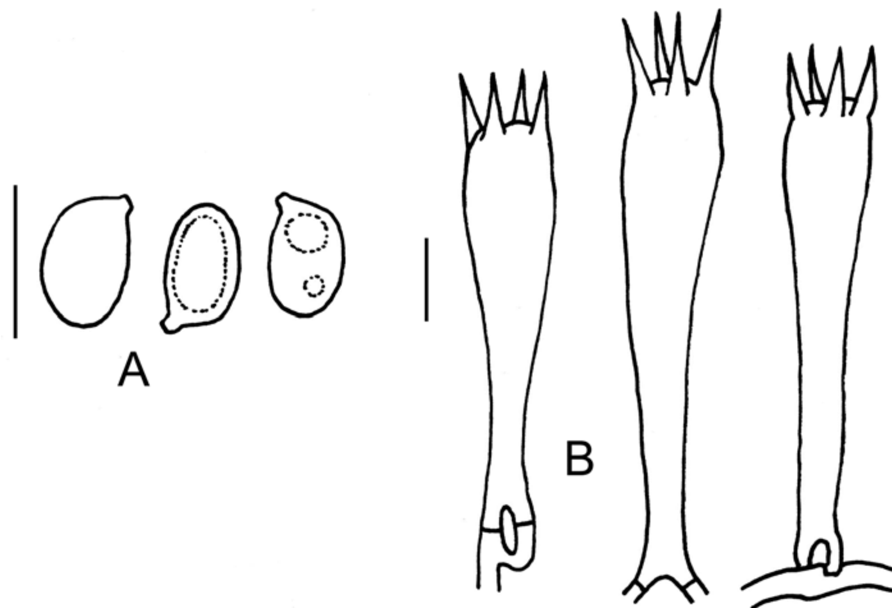

Figure 3. *Humidicutis viridimagentea*. A, basidiospores showing clear inclusions; B, basidia from left to right displaying: a medallion clamp that has almost become toroidal, a fractured base resulting from disintegration of a toroidal clamp, and a basidium emerging directly from a horizontal hypha. Scale bars = 10 µm.

septate hyphae 1-2 µm diam., clamp connections absent.

Habitat: Amongst moss underneath bracken (*Pteridium esculentum*) in eucalypt woodland (*Eucalyptus patens*, *Agonis flexuosa*); gregarious and occasionally caespitose.

Other material: Western Australia. Denmark, 34°58′44″S 117°13′52″E, 9.vi.2004, *K. Syme 1335/04*, (MEL 2279341).

Remarks: *Humidicutis viridimagentea* is similar to other green coloured Australasian taxa in the genus but differs in its very distinctive magenta coloration. There are two similar New Zealand taxa. *Humidicutis luteovirens* (E. Horak) E. Horak is at first green with yellow lamellae, however the entire basidioma slowly changes to yellow with age; and *H. multicolor* (Berk. & Broome) E. Horak has olive green lamellae which slowly change to lilac or blue and slightly smaller spores (5.5-7.0 × 4.5-5.0 µm) (Horak 1990). Three known Australian taxa also possess green pilei, however

Humidicutis arcohastata (A.M. Young) A.M. Young (Young 2005) slowly exhibits bright orange tints or may become wholly bright orange and has acute hyphal endings in the pileipellis; *H. helicoides* (A.M. Young) A.M. Young (Young 2005) has fusiform hyphae in the pileipellis which exhibit helical banding of the hyphal walls; and *H. taekeri* has orange lamellae and larger ellipsoidal to subglobose spores (5.0-9.5(-10.5) × 4.0-6.5(-7.5) µm). None of these Australian taxa exhibits the magenta colourations in either fresh or dried material (Young 2005).

The second collection of this species (MEL 2279341) differs from the holotype in that it contains basidiomata that have mostly 2-spored basidia. These 2-spored basidia have the same size and basal structure as the 4-spored basidia of the holotype but the sterigmata are much longer and can be up to 12 µm in length. The basidiospores are also larger: (6.5-)7.2-10.1(-11.5) × 4.7-7.2 µm, mean 8.6 × 6.0 µm, Q: 1.2-1.7(-1.9), mean Q: 1.45, but have the same shape and additionally the single large inclusion. Such 2-spored forms are well known from other species of the Hygrophoraceae such as *Hygrocybe virginea* (Wulfen:Fr.) P.D. Orton & Watling or *Hygrocybe acutoconica* (Clem.) Singer.

An interesting aspect of *H. viridimagentea* is that the cap and stipe surfaces of fresh, green material become dull magenta (near 8C6-8D6) during the drying process. Other green coloured members of the Hygrophoraceae also exhibit a colour change during drying but become dull brick pink (near 6A3): *Hygrocybe graminicolor* (E. Horak) T.W. May & A.E. Wood and *H. stevensoniae* T.W. May & A.E. Wood. This suggests some difference or differences in chemical composition of the pigments in the relevant groups.

The basidial clamp connections in *H. viridimagentea* are often so highly modified as almost to prevent their identification as such. During the rehydration and mounting of dried material, the toroidal clamps frequently disintegrate and only a "Y-shaped" base remains on the basidium (Young 2005). Other basidia emerge directly from more or less 'horizontal hyphae' and the only evidence of the modified clamp connection is the "Y-shaped" base with the space between the basidium and the parent hypha.

Acknowledgements

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VISUALISING MACROFUNGAL SPECIES ASSEMBLAGE COMPOSITIONS USING CANONICAL DISCRIMINANT ANALYSIS

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Abstract

Recent generalisations of canonical variate analysis allow the technique to be applied to typical ecological data sets such as the presence or absence, or the abundance, of each species in a long list of species. This provides a useful tool for testing and visualising differences in assemblage compositions obtained from macrofungal surveys. In this paper, one such advance, the canonical analysis of principal coordinates (CAP), is illustrated using data sets obtained from macrofungal surveys conducted in the silvicultural treatment areas of the Warra long-term ecological research site in southern Tasmania, Australia. Differences in species assemblage compositions are tested using non-parametric permutation tests that provide exact probability values, and the similarity or dissimilarity of the various sampling units are graphically displayed in a manner easily understood by ecologists. The CAP ordination diagrams in the two illustrative examples clearly display the differences in the mycota between the earliest stage of regeneration and the mature forest, and their associated statistical tests quantify these differences.

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Introduction

Ordination techniques aim to display graphically the differences between experimental units in data sets involving two or more variables. The variables may be of a variety of types: measures or counts, continuous or discrete, ordered or disordered, etc. The units may have been sampled randomly or opportunistically, or may have been subjected to different treatment regimes. Conveniently, ordination techniques may be classified as unconstrained or constrained. Unconstrained ordinations include multidimensional scaling, whether non-metric (nMDS) or metric, the latter including principal component analysis (PCA), which is based upon a Euclidean distance measure, principal coordinate analysis (PCoA), a generalisation of PCA that allows other definitions of distance (or dissimilarity) such as ones in use in ecology, and correspondence analysis, whether detrended (DCA) or not (CA). These unconstrained ordinations share the common feature that the experimental units are unstructured, i.e. they are not divided into groups and, furthermore, they do not incorporate any null hypotheses that can be tested statistically. Despite these restrictions, they still may display the variation that exists within a cloud of data points, and perhaps suggest hypotheses that may be subjected to statistical tests by other procedures.

Constrained ordinations, by comparison, operate on structured experimental units, which may typically be predefined groups. One standard ordination technique used by biometricians for testing and displaying differences between predefined groups of experimental units is multivariate canonical variate analysis (CVA). When coupled with the associated feature of discriminant analysis, CVA can assign new individuals (i.e.,

experimental units) to the group to which they are closest, using discriminant functions based upon a linear combination of a set of explanatory variables. Traditionally, these statistical techniques have necessarily been limited to variables that follow multivariate normal distributions, making them unsuitable for ecological data arising from inventory sampling, where the presence or absence, or abundance, of each species in a list of species often forms the raw data set. A further constraint imposed by the traditional CVA is that the number of variables must be less than the number of observations. This may not apply to ecological data sets where the variables are species, the lists often being many times longer than the number of observations (e.g., the total number of visits made to the sampling units).

Anderson and Willis (2003) recognised the need for a flexible method of constrained ordination that could be applied to any distance or dissimilarity measure, so that the limitations imposed by the traditional CVA could be overcome. This was achieved by coupling metric principal coordinate analysis (PCoA), which allows any definition of distance or dissimilarity, with CVA to produce canonical analysis of principal coordinates (CAP). That coupling leads to two pathways, one of which is a canonical discriminant analysis, which tests hypotheses concerning groups, and the other is a canonical correlation analysis, which tests hypotheses regarding relationships with additional sets of quantitative predictor variables such as environmental or genetic factors or measures. It is the first use of CAP with which the present communication is concerned. The mathematical details behind CAP are given in Anderson and Robinson (2003), whereas the ecological perspective is provided in Anderson and Willis (2003). Both papers offer practical examples drawn from sampling fish assemblages in a marine reserve. To date, applications in which CAP has been used for data on fungi are rare, although two recent papers (Bastias *et al.* 2006a, 2006b) applied CAP to data obtained from fungal DNA from forest plots that had different prescribed burning treatments to test whether the burning regimes altered soil and ectomycorrhizal fungal community structure. In the present paper, examples are drawn from macrofungal surveys in forests subjected to different silvicultural treatments and the examples contrast unconstrained and constrained ordinations of the data. In particular, CAP tests the null hypothesis that there are no differences between fungi in forests subjected to different silvicultural treatments or visited at different times of the year.

Materials and Methods

The present paper illustrates the application of CAP to macrofungal species assemblages. It contrasts the ordination diagrams from CAP with those from an unconstrained ordination, PCoA. Although other unconstrained ordinations could be used, for example nMDS, the latter is not an objective procedure, relying on random starting points and a selection of one solution amongst many based upon the minimisation of the "stress" criterion. This lack of objectivity means that different implementations of nMDS may result in different ordination diagrams. In contrast, PCoA as originally devised by Gower (1966) is an objective procedure that should produce the same ordination diagrams irrespective of which computer package computes them. Moreover, PCoA is the starting point for a CAP analysis, so that a single computer run will produce both a PCoA and CAP analysis if the appropriate options are chosen.

The examples are drawn from two surveys conducted at the Warra LTER (long-term ecological research) site in southern Tasmania. The first survey involved a comparison between macrofungal species in a regenerating coupe after a clearfelled, burnt and sown (CBS) silvicultural treatment had been applied, and in a mature forest in close proximity to the CBS coupe (Gates *et al.* 2005). 307 species were found in the two coupes. The second survey also involved a comparison of silvicultural treatments, in this case between 387 macrofungal species found collectively in an "aggregated retention" coupe and in a mature forest (Gates and Ratkowsky 2006). In this aggregated retention coupe, the trees in ca. 70% of the area were harvested, followed by a low intensity burn to stimulate regeneration. No artificial sowing of seeds took place. The remaining 30% of the area was retained in eight islands, of which three were

Table 1. Number of visits to the experimental units of each coupe in each of the two surveys (for more details, see Gates *et al.* 2005, Gates and Ratkowsky 2006, respectively).

Survey	Autumn (Mar-May)	Winter (Jun-Aug)	Spring (Sep-Nov)	Summer (Dec-Feb)
First	7	7	7	6
Second	13	8	7	7

surveyed for fungi. Separate lists were made of the fungi in the harvested and unharvested portions of the aggregated retention coupe, so that together with the mature forest, three distinct groups of experimental units could be compared. For comparability, the total length of the transect within the three groups was approximately equal. It was hypothesised that the fungi in the harvested portions of the aggregated retention coupe would be very distinct from the fungi in the unharvested islands and in the mature forest, with the latter two units showing some degree of similarity as the mature forest and the aggregated retention coupe were adjacent to one another and were of the same forest type.

In both surveys, multiple visits were made in all four seasons, providing some degree of replication, although the replication was pseudoreplication (see Hurlbert 1984) and an unequal number of visits was made. Nevertheless, this replication made it possible to test for coupe differences or seasonal differences using CAP by means of a permutation test and also to test for misclassifications using its "leave-one-out" allocation procedure. Table 1 summarises the number of visits made during each season. In both surveys, visits to each sampling unit in each coupe were made on the same day, thereby eliminating biases that would occur had this not been possible.

In both surveys, fungal species records from each visit were converted to presence/absence and Bray-Curtis dissimilarities were used, without standardisation or transformation.

Results

First Warra survey:

Figs 1 and 2 display the first two principal coordinate axes of a PCoA analysis and the first two canonical axes of a CAP analysis for the first Warra survey, respectively. The first axis in both diagrams clearly separates the fungal assemblages in the two coupes, with the points from the harvested coupe (H) all having positive scores, and the points from the mature forest (M) all having negative scores. The second axis in both procedures has only partial success in separating the sampling units with respect to seasonal effects, but almost succeeds for the mature forest using the CAP analysis. Plotting the third canonical axis from CAP (not shown) confirms the separation of the assemblage compositions in the four seasons within the mature forest, as the third axis clearly separates the spring from the summer results. Within the harvested coupe, the separation remains unclear. Note that in some instances there are fewer points in Figs 1-2 for a combination of coupe and season than the number of visits given in Table 1 indicate, e.g. four points for the regenerating coupe in summer compared to six visits actually made. This was caused by the fact that there were no fungi observed during some visits to that coupe, these "zero lists of species" occasionally occurring during the drier months.

The PCoA axes presented in Fig. 1 display information about the similarities between individual visits, but do not quantify differences between groupings. As PCoA is unconstrained, there are no tests of significance embodied in the procedure. CAP, on the other hand, provides an exact test of the null hypothesis that there are no differences between the eight groups formed by combining coupe and seasonal effects, using random permutations. The P-value obtained from the permutation test performed by CAP was P=0.00020 using 4999 permutations. Since 1/(4999 + 1)=0.00020, this means that no randomly permuted data set had a more contrastingly different macrofungal species assemblage than that of

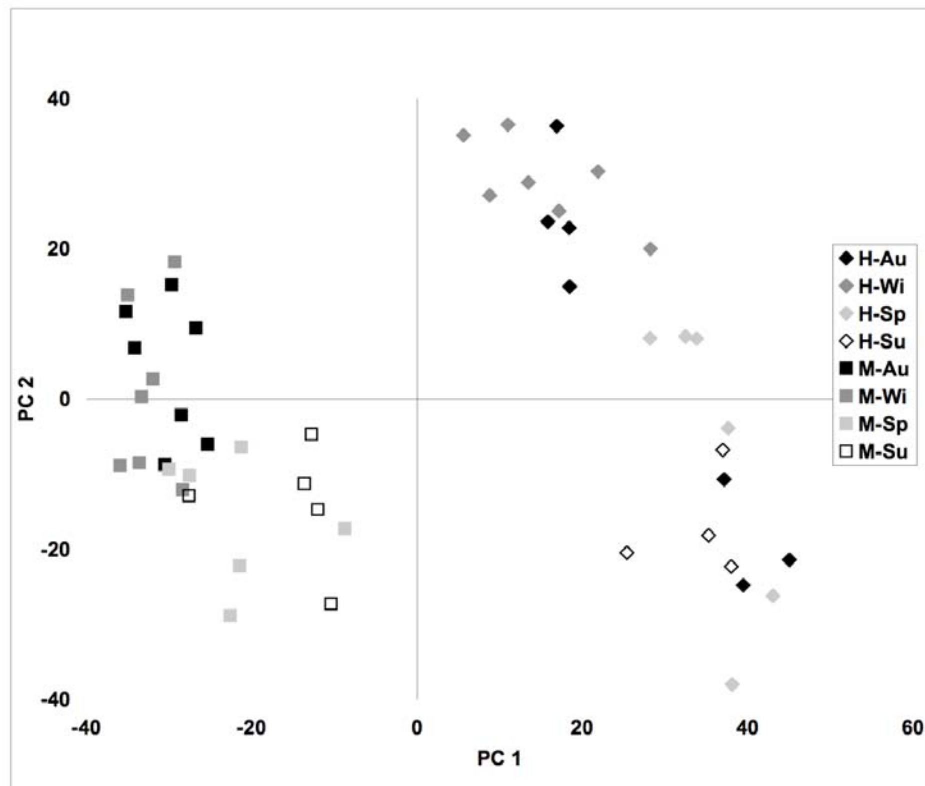

Figure 1. The first two principal coordinates of the PCoA analysis, first Warra survey.

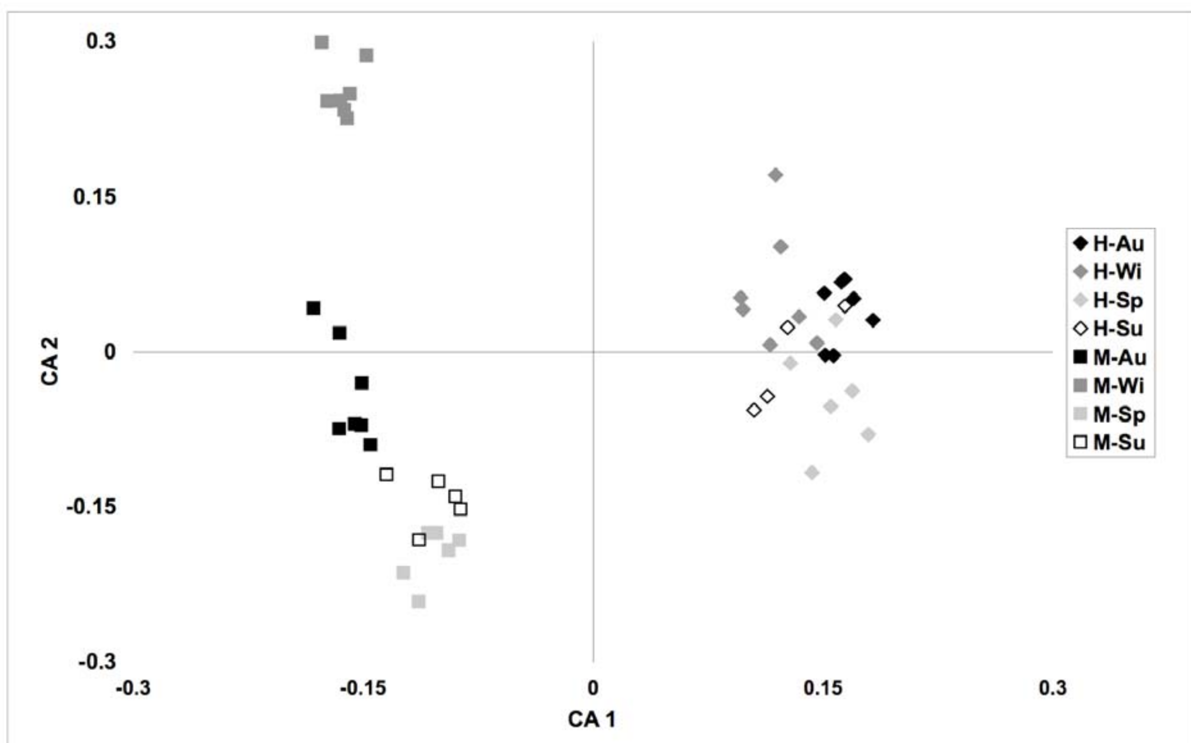

Figure 2. The first two canonical axes of the CAP analysis, first Warra survey.

[Note: In Figs 1 & 2, the first letter represents the plot (H=CBS coupe; M=mature forest) and the next two letters represent season (Au=autumn; Wi=winter; Sp=spring; Su=summer)].

Table 2. Classification table, first Warra survey, showing the reclassification of the original visits into groups, and the success and misclassification rates of the CAP analysis.

Original group	Classified into groups									
	HAu	HWi	HSp	HSu	MAu	MWi	MSp	MSu	Total	%correct
HAu	4	1	1	1	0	0	0	0	7	57.1%
HWi	0	7	0	0	0	0	0	0	7	100%
HSp	1	0	4	1	0	0	0	0	6	66.7%
HSu	0	0	0	4	0	0	0	0	4	100%
MAu	0	0	0	0	7	0	0	0	7	100%
MWi	0	0	0	0	0	6	1	0	7	85.7%
MSp	0	1	0	0	0	1	4	0	6	66.7%
MSu	0	0	0	0	1	0	0	4	5	80.0%
			Total correct = 40/ 49 = 81.6%							
			Misclassification error = 18.4%							

[Note: The first letter represents the plot (H=CBS coupe; M=mature forest) and the next two letters represent season (Au=autumn; Wi=winter; Sp=spring; Su=summer)].

Table 3. Selected list of species occurring at least seven times and only in one or other of the two coupes, first Warra survey.

Species exclusive to the Clearfelled, Burnt, Sown regenerating coupe	Species exclusive to the unharvested mature forest
Bovista brunnea Berk.	*Ascocoryne sarcoides* (Jacq.) J.W.Groves & D.E.Wilson
Galerina nana (Petri) Kühner	*Auriscalpium* 'warrensis'
Gerronema 'pink-buff'	*Chondrostereum purpureum* (Pers.) Pouzar
Gymnopus 'dark brown, hygrophanous'	*Dermocybe kula* Grgur.
Psilocybe 'dark brown, pellucid, in moss'	*Galerina* 'small, umbonate with sphaeropedunculate cheilocystidia'
Pycnoporus coccineus (Fr.) Bondartsev & Singer	*Hydnum repandum* L.: Fr.
Schizophyllum commune Fr.:Fr.	*Lactarius clarkeae* Cleland
Scutellinia aff. *margaritacea* (Berk. ex Cooke) O.Kuntze	*Lactarius stenophyllus* Berk.
Stereum ochraceoflavum (Schwein.) Sacc.	*Marasmiellus affixus* (Berk.) Singer
Trametes versicolor (L.:Fr.) Lloyd	*Mycena albidocapillaris* Grgur. & T.W.May
	Phellodon niger (Fr.: Fr.) P.Karst.
	Ryvardenia campyla (Berk.) Rajchenb.

the original data set. Therefore, one would be justified in concluding that there are at least some significant differences among the eight groups. Using a leave-one-out approach (see Appendix A of Anderson and Willis 2003 for the details), one can take each of the original visits in turn and determine to which of the eight groups its fungal assemblage is closest. The result of this reclassification is presented in Table 2. Only one misclassification occurred between coupes, with one of the spring visits to the mature forest being classified as a winter visit to the regenerating coupe. All the other misclassifications occurred within the same coupe, with some assignments of the seasons being erroneous. Overall, the classification success rate was 81.6% (Table 2). For completely random data, the classification success would be expected to be

only 100/8 = 12.5%, suggesting that the groups are different.

Some of the more notable differences between the species of fungi in the two coupes are summarised in Table 3, which includes a selection of the most frequently occurring species that were found only in one or the other of the two coupes. The entries in each column comprise species that occurred exclusively within the coupe indicated, and were obtained directly from lists of the 307 fungal species in the database. Alternatively, one may employ the correlations between each of the species and the canonical axes that are part of the output of CAP to assist in interpreting the axes. However, such correlations are often not readily interpretable, as the axes maximise the separation between all eight groups, not just the major groupings, and recourse to the original data is often a better option. A full list of the 307 fungal species may be found in Gates *et al.* (2005).

Second Warra survey:

For the data set from the second Warra survey, Figs 3 and 4 display the first two principal coordinate axes of a PCoA analysis and the first two canonical axes of a CAP analysis, respectively. In both ordinations, the first axis clearly separates the harvested portions (H) of the aggregated retention coupe from the unharvested islands (U) and from the mature forest (M), but the two uncut areas are not separated on that axis. The second principal coordinate axis of PCoA (Fig. 3) does not succeed in separating those sampling units, but the second canonical axis of CAP (Fig. 4) appears to largely separate them, a fact that can be confirmed from an examination of the classification matrix (Table 4), which uses a leave-one-out allocation procedure to determine to which of the 12 experimental units (i.e. combinations of the parts of the coupe and season) each species list is the most similar.

From Table 4, it is seen that misclassifications among the major sampling units are rare, with only two visits being reclassified wrongly to a major sampling unit. No misclassifications of visits to the harvested coupe (H), or to the mature forest (M), occurred, the two misclassifications being for visits to the unharvested portion (U) of the aggregated retention coupe, both of which were mistakenly reclassified by the discriminant functions as visits to the mature forest. Aside from these two, all other misclassifications were between seasons within the same coupe. As the total misclassification error was 34%, erroneous assignments to season were more frequent than in the first Warra survey.

The P-value obtained from the permutation test performed by CAP was P=0.00020 when the number of permutations used was 4999, so that, in common with the first Warra survey, no randomly permuted data set had a more extreme macrofungal species assemblage than that of the original data set.

Table 5 summarises some of the most notable differences between the species of fungi in the two coupes, the list including a selection of the most frequently occurring species. The entries in the first column comprise species that occurred only in either the mature forest or the uncut portion of the aggregated retention coupe, or both, whereas the second column lists species that occurred only in the harvested areas of the aggregated retention coupe. The full list of 387 macrofungal species, and the coupes, habitats and substrates on which they occurred, is given in Gates and Ratkowsky (2006).

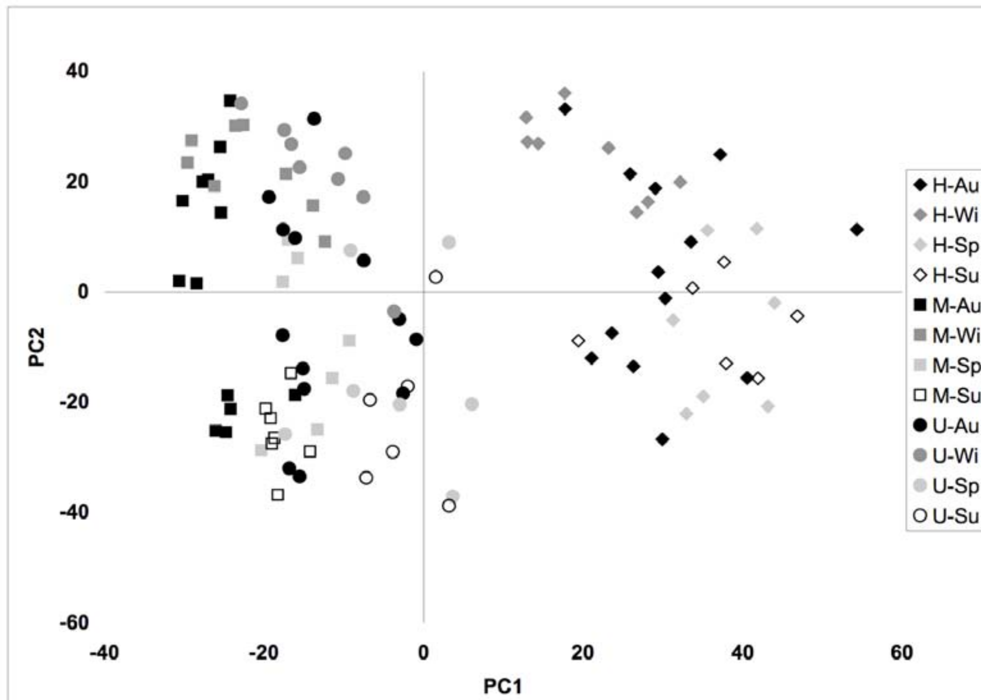

Figure 3. The first two principal coordinates of the PCoA analysis, second Warra survey.

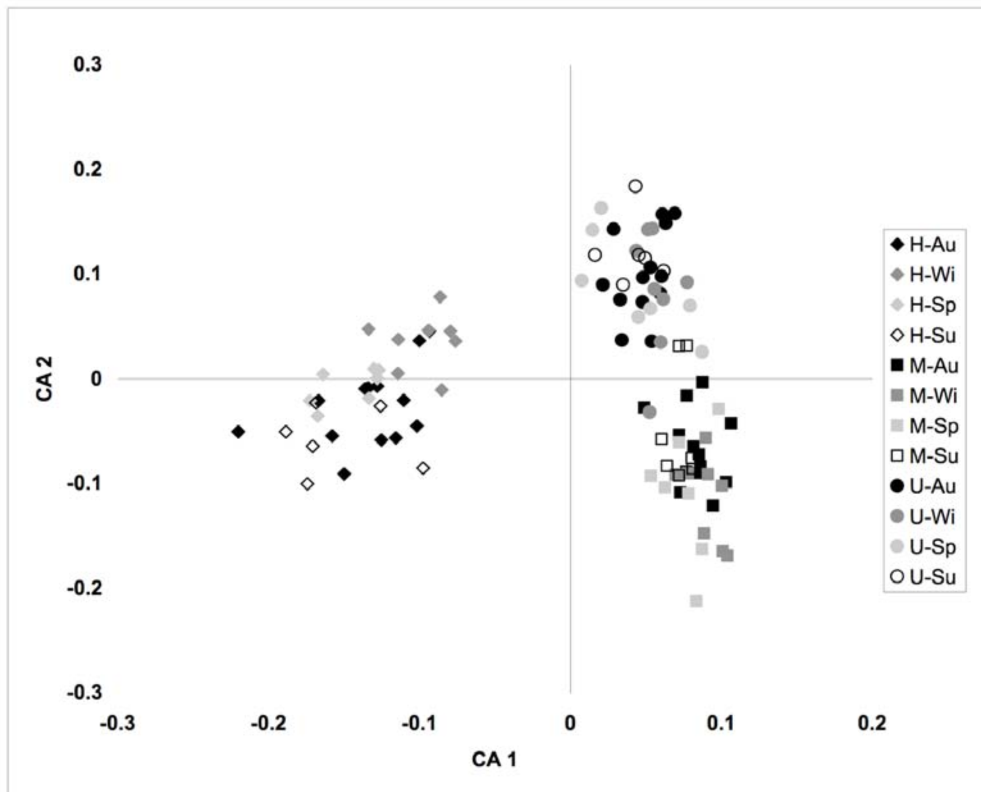

Figure 4. The first two canonical axes of the CAP analysis, second Warra survey.

[Note: In Figs 3 & 4, the first letter represents the plot (H=harvested portion of aggregated retention coupe; M=mature forest; U=unharvested portion of aggregated retention coupe) and the next two letters represent season (Au=autumn; Wi=winter; Sp=spring; Su=summer)].

Table 4. Classification table, second Warra survey, showing the reclassification of the original visits into groups, and the success and misclassification rates of the CAP analysis.

Original group	Classified into groups												
	HAu	**HWi**	**HSp**	**HSu**	**MAu**	**MWi**	**MSp**	**MSu**	**UAu**	**UWi**	**USp**	**USu**	**Total**	**%correct**
HAu	6	2	1	4	0	0	0	0	0	0	0	0	13	46.2%
HWi	0	8	0	0	0	0	0	0	0	0	0	0	8	100.0%
HSp	1	1	3	2	0	0	0	0	0	0	0	0	7	42.9%
HSu	0	0	2	4	0	0	0	0	0	0	0	0	6	66.7%
MAu	0	0	0	0	11	1	0	1	0	0	0	0	13	84.6%
MWi	0	0	0	0	1	7	0	0	0	0	0	0	8	87.5%
MSp	0	0	0	0	0	1	3	3	0	0	0	0	7	42.9%
MSu	0	0	0	0	0	0	1	6	0	0	0	0	7	85.7%
UAu	0	0	0	0	1	0	0	0	9	1	1	1	13	69.2%
UWi	0	0	0	0	0	0	0	0	1	5	2	0	8	62.5%
USp	0	0	0	0	0	0	1	0	0	1	3	2	7	42.9%
USu	0	0	0	0	0	0	0	0	3	0	0	3	6	50.0%

Total correct = 68/103 = 66.0%
Misclassification error = 34.0%

[Note: The first letter represents the plot (H=harvested portion of aggregated retention coupe; M=mature forest; U=unharvested portion of aggregated retention coupe) and the next two letters represent season (Au=autumn; Wi=winter; Sp=Spring; Su=summer)].

Table 5. Species occurring at least 10 times and only in the mature forest and/or in the uncut parts of the aggregated retention coupe versus those in the harvested parts of the aggregated retention coupe, second Warra survey.

Species exclusive to the mature and/or uncut forest	Species exclusive to the harvested areas
Boletellus obscurecoccineus (Höhn.) Singer	*Aleuria aurantia* (Pers.) Fuckel
Cantharellus concinnus Berk.	*Byssomerulius corium* (Pers.: Fr.) Parmasto
Cortinarius 'C48, lilac and brown, *Phlegmacium*'	*Coprinellus angulatus* (Peck) Redhead, Vilgalys & Moncalvo
Cortinarius 'C62, varnished, golden brown with sharp reddish umbo'	*Galerina nana* (Petri) Kühner
Entoloma austroprunicolor G. Gates & Noordel.	*Loreleia marchantiae* (Singer & Clémençon) Redhead, Moncalvo, Vilgalys & Lutzoni
Galerina 'small, umbonate with sphaeropedunculate cheilocystidia'	*Lyophyllum* 'small, brown'
Lactarius clarkeae Cleland	*Mycena* 'brown striate, becoming sulcate'
Lactarius eucalypti O.K. Mill. & R.N. Hilton	*Pholiota highlandensis* (Peck) A.H. Sm. & Hesler
Marasmiellus affixus (Berk.) Singer	*Pycnoporus coccineus* (Fr.) Bondartsev & Singer
Mycena interrupta (Berk.) Sacc.	*Schizophyllum commune* Fr.: Fr.
Mycena toyerlaricola Grgur.	
Phellodon niger (Fr.: Fr.) P. Karst.	
Pholiota squarrosipes Cleland	
Podoserpula pusio (Berk.) D.A. Reid	
Pulveroboletus ravenelii (Berk. & M.A. Curt.) Murrill	
Ryvardenia campyla (Berk.) Rajchenb.	
Stereum ostrea (Blume & Nees: Fr.) Fr.	

Discussion

In the two examples from macrofungal surveys at the silvicultural treatment trials in the Warra LTER in southern Tasmania, there was a large measure of agreement between the PCoA and CAP ordination diagrams. Both techniques succeeded in visualising the separation between the major experimental units but were more limited in separating seasons. The latter result is not surprising, given the arbitrary nature of the boundaries between seasons, the seasons being based upon a contiguous three months' period (Autumn: March-May; Winter: June-August; Spring: September-November; Summer: December-February). However, CAP does more than just provide an ordination diagram, expressed as canonical axes. By incorporating a permutation test, the user can test the null hypothesis of no difference between the macrofungi lists in the sampling units and obtain an exact, distribution-free P-value. In each of the illustrative examples, 4999 random permutations were used, and a P-value of 0.00020 resulted. This is interpreted as meaning that the differences among the macrofungal assemblages in the eight sampling units of the first trial and the 12 sampling units of the second trial are real and not due to chance.

The canonical discriminant analysis procedure applied by CAP maximises the differences among the predetermined structural units and provides a test of significance, unlike nMDS, PCoA, DCA and other unconstrained ordination procedures, where the data are unstructured. Although there may seem to be little justification for use of an unconstrained ordination when the experimental design has a clearly defined structure and one has an explicit multivariate hypothesis to test *a priori*, Anderson and Willis (2003) argue that there may be additional patterns in the multivariate data cloud that are not detected by CAP. Therefore, they suggest that a robust unconstrained ordination be used in conjunction with the constrained CAP analysis. This recommendation has been followed here, and the similarity between the CAP and PCoA diagrams suggests that CAP is capturing the most important factor influencing the assemblage structure.

In this paper, PCoA was chosen in preference to nMDS to illustrate the unconstrained ordination procedure, as it is an objective method of MDS and is available as an option when using CAP. To compare the results to those from a nonmetric procedure, the first two axes of a three-axes nMDS ordination of the data from the second Warra survey, carried out using Primer 6 (2006), are presented in Fig. 5. The original data matrix is the same as that for the CAP analysis presented in Fig. 3, i.e. presence/absence data were used, not transformed and not standardised, with Bray-Curtis dissimilarities.

In Fig. 5, the separation between the cloud of points for the harvested portion (H) of the aggregated retention coupe and that for the mature forest (M) is very clear, but the overall interpretation of the ordination is muddied by the very wide scatter of the data points for the unharvested portion (U) of the aggregated retention coupe. Aside from being located in more or less the right position, i.e. somewhere between the clouds of points for the other two treatments, there is little else to be learned from this diagram. Seasonal effects are also not revealed, with points for each season being spread over a wide range. Displaying the third axis (not shown here) of the three-axes solution does not add anything further to the interpretation.

Some further remarks

In the first CAP example presented here (see Fig. 1), the spread of the points within the mature forest (M) and within the clearfelled, burnt and sown group (H) appear to suggest that the distances between the observations, and hence the variability, is greater for the control group. However, such a conclusion would be unjustified without carrying out a formal statistical hypothesis test, as the distances displayed on a canonical variates diagram are not Euclidean distances, but Mahalanobis distances (see Legendre and Legendre 1998). That is, the distances between points take account of the variances and covariances of the species. The consequence of this is that differences in dispersion cannot be deduced from how the points are arranged on the axes of a CAP diagram, but must be tested using a formal statistical test. A procedure (PERMDISP2) for

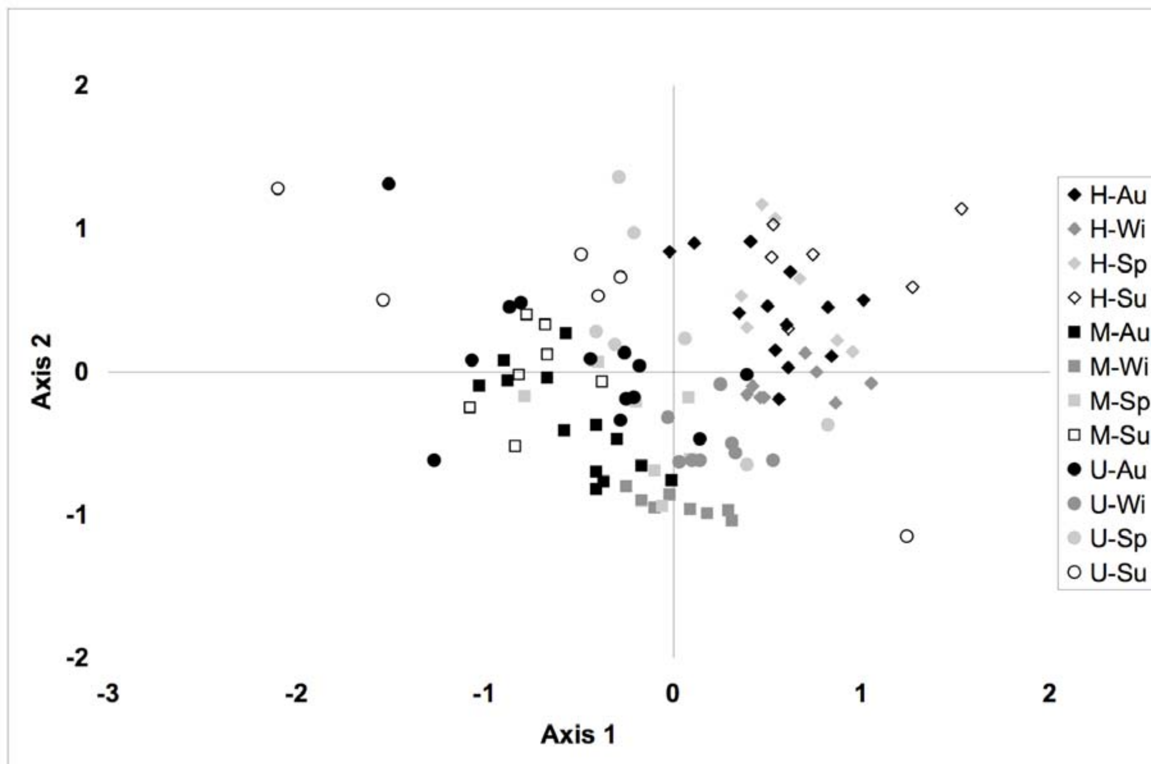

Figure 5. Axis 2 vs. Axis 1 of a nMDS analysis of macrofungal data, second Warra survey.

[Note: The first letter represents the plot (H=harvested portion of aggregated retention coupe; M=mature forest; U=unharvested portion of aggregated retention coupe) and the next two letters represent season (Au=autumn; Wi=winter; Sp=spring; Su=summer)].

doing this has been developed by M.J. Anderson, which performs a distance-based permutation test for the homogeneity of multivariate dispersions in groups of unequal sample sizes and is based upon any definition of similarity (such as Bray-Curtis).

CAP is available for downloading as freeware from M.J. Anderson's home page at the University of Auckland. The address is

http://www.stat.auckland.ac.nz/~mja/

User notes explaining step-by-step how the programs are used may also be downloaded from that website.

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SOME OBSERVATIONS ON *CRINIPELLIS AUSTRALIS*

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Abstract

Crinipellis australis has been found at several locations in the city of Canberra, Australian Capital Territory (ACT). The species was previously known only from Sydney and Adelaide. The Canberra finds suggest the species has a preference for *Themeda australis* and is able to exploit irregular rainfall. This paper will supplement the previously published macroscopic description.

H. Lepp (2007). Some observations on *Crinipellis australis. Australasian Mycologist* 26 (2-3): 86-90.

Introduction

Crinipellis australis Grgurinovic was described by Grgurinovic (1997) who listed three herbarium collections, gathered from Sydney and Adelaide at around the time of the First World War. May *et al.* (2004) record no other published finds of this species and note that the only other publication to mention the species is Grgurinovic & Simpson (2001), who classified the taxon as "poorly known". The work leading to this paper started because of curiosity about some small brown agarics growing near the base of a *Themeda* tussock in a remnant native grassland area in suburban Canberra. Later microscopic examination showed them to be *Crinipellis australis* and this find led to a search for the species in other grassy habitats in Canberra. The search proved successful, with many specimens sighted and several collections now deposited at the Australian National Herbarium (acronym CANB) at the Centre for Plant Biodiversity Research in Canberra. A search at CANB turned up one additional collection, from 1991, amongst the unidentified agarics.

Some features of the Canberra specimens

Cleland (1934) gave a macroscopic description, but under the name *Crinipellis caulicinalis* (Bull.) Rea. Grgurinovic (1997) repeated Cleland's macroscopic description and added descriptions and drawings of microscopic features. The Canberra finds gave an opportunity to record some supplementary information about the macroscopic features (Table 1).

Habit: In Canberra sporocarps grew separately or in small caespitose clusters. on or near grasses. Grgurinovic (1997) noted they were "gregarious on the ground, often attached to buried grass stems".

Microscopic features: The Canberra collections add nothing to the microscopic detail recorded by Grgurinovic (1997). She noted that the basidiospores are "rarely septate" and I saw but one clearly septate spore. She noted that the thick-walled pileal and stipe hairs are only slightly dextrinoid and I fully agree with that finding. The dextrinoid reaction may be very slow in showing and even after a long time is usually still quite weak. There may also be variation within a specimen. For example, in one sporocarp from collection *H Lepp 551* the stipe hairs showed an easily observable, mildly dextrinoid reaction whereas the pileal hairs varied from no observable reaction to at most very weakly dextrinoid.

Table 1.

Macroscopic information given by Grgurinovic (1997)	Supplementary information derived from the Canberra collections
Pileus to 12 mm diam; irregularly convex, covered with intricate appressed fibrils, edge sometimes slightly rugose, pallid brownish drying to a pale greyish brown or dead grass colour	Pilei are up to 15 mm in diameter and covered with short, dense, radially orientated fibrils. The pilei are hemispherical (or slightly flattened-hemispherical) when young but with age become convex to shallowly convex or, occasionally, nearly plane. In the convex specimens there may be a brief, turned out (or even slightly upturned) flange. Sometimes there is a slight central papilla, generally best seen in a pileus beginning to dry out. The pilei show various brownish shades, from greyish orange (near 6B4/6B5 and 6C3/6C4) through greyish brown to reddish brown (near 8D3/8D4). Within any population the younger pilei are darker, some dark brown (9F6 to 9F8). The marginal flange, where present, is paler than the rest of the pileus. In very immature pilei the margins may be white and markedly woolly.
Lamellae apparently adnate or adnexed to an indefinite collar, then seceding, moderately close to concolorous	Colours are greyish red (near 7B3/8B3), various shades of greyish brown to dull red (near 9B3/9C3). The margins may be weakly crenulate. The collar noted by Ggrurinovic (1997) varies from indistinct to well-developed.
Stipe up to 25 mm long, slender, usually attenuated downwards, densely pilose, solid, concolorous with the pileus or sometimes browner	Stipes grow to 30 mm long and 1-2 mm diameter and are even or slightly tapered downward. They are pilose, generally densely so but only patchily so in some cases – presumably through loss of hairs by abrasion. The stipes are in shades of brown – from reddish brown, through brown to dark brown (from 7E6/8E6 to high 9F shades) but generally paler towards the apices, at times closely approaching the pileus in colour.

All bracketed colour references are to Kornerup & Wanscher (1989).

Specimens studied:

AUSTRALIAN CAPITAL TERRITORY. Barton: on the dead parts of *Themeda* tussocks; in remnant *Themeda* grassland, with scattered eucalypts; 17 November 2004; *H Lepp 4605*. **Black Mountain Nature Reserve**: on grass roots and shafts; in a grassy clearing beneath powerlines through dry sclerophyll woodland; 9 June 1991; *H Lepp 551*. **Bruce**: on dead grass tussocks; in an exposed grassy slope, with scattered eucalypts; 27 November 2004; *H Lepp 4620*. **Campbell**: on dead grass or on soil; in remnant native grassland; 14 December 2004; *H Lepp 4632*. **Cook**: on dead material at bases of *Themeda* tussocks, on totally dead tussocks or from buried vegetable matter (?grass roots); in *Themeda*-dominated grassland with scattered eucalypts; 20 November 2004; *H Lepp 4612*. On dead material at bases of *Themeda* tussocks or from buried vegetable matter (?grass roots); in open grassy area with scattered eucalypts; 13 December 2004; *H Lepp 4628*. **Cook, Canberra Nature Park**: on dead material at bases of *Themeda* tussocks; in *Eucalyptus* woodland, with an understorey of shrubs and grasses; 20 November 2004; *H Lepp 4613*. **Glenloch**: on

dead parts of grass tussocks or from buried vegetable matter (?grass roots); in a patchily grassed grazing paddock (with *Themeda*, *Austrodanthonia* or *Nothodanthonia*, *Vulpia*) and scattered eucalypts; 15 December 2004; *H Lepp 4635*.

Comments about the collection sites

The site altitudes are between 500 and 650 metres above sea level. Apart from the two sites in the suburb of Cook, the sites are at least two kilometres away from each other and all are contained within a circle having a diameter of about 10 kilometres.

Barton: This is a 1.9 hectare area that is registered as site CC04 in ACT Government (2005). Despite its small size it has a high conservation value since it is an area of dry *Themeda* grassland that has been little disturbed by exotic weeds or by human activity. It has been given a Botanical Significance Rating of "very high", the highest used in the previously-cited reference. There are nearby office blocks and the area does attract lunchtime walkers and joggers, but such traffic appears to be always along footpaths around the perimeter.

Black Mountain Nature Reserve: Tall vegetation had been removed from beneath the power lines. The remaining vegetation was mostly grass (native and exotic), with some scattered shrubs and the occasional *Acacia* or *Eucalyptus* - regrowth from stumps or fresh saplings that had grown from seed since the last clearance work under the power lines. The clearance work is done irregularly at intervals of many years.

Bruce: The site is an undeveloped area in the grounds of the University of Canberra. It is on a moderate slope with clay/gravelly soil, mostly grassed but with some eucalypts. It is likely to be mown annually to reduce fire risk, but otherwise would see little human visitation, not being close to any major student facilities.

Campbell: This is site CC02 in ACT Government (2005), where it is mistakenly allocated to the suburb of Reid. It has a "moderate" Botanical Significance Rating and has a high cover of native grass but with a low to moderate level of exotic species and a moderate level of human disturbance. Along one edge of the area there is a well-worn foot track that is used by people going to or from nearby houses. It is likely that the area is also used by people exercising their dogs.

Cook: This is an area of about 500 by 200 metres, on a moderate slope and largely grassy, with a small number of eucalypts. The grass cover is not homogenous, varying both in density of cover and species composition. There is a small patch of almost pure *Themeda* at the top of the slope, through a mixture of *Themeda*, other native grasses and some exotic species to almost purely exotic species at the lowest point of the slope, with no *Crinipellis* sporocarps found in the lowest section. The area has a bitumen cycle path through it that is used by both commuters and recreational cyclists. The area is also used by people walking their dogs or children occasionally playing ball games and would be mown once or twice a year.

Cook, Canberra Nature Park: This is a woodland area, on a steeper slope in which there is a fairly dense cover of native grasses and shrubs between the eucalypts. This woodland patch would rarely see any human visitation.

Glenloch: The paddock has a long history of grazing and carries low numbers of cattle from time to time but is not constantly stocked. The area carries native and exotic grasses as well as other exotic weeds.

General comments

Cleland (1934) wrote that the species grew "on the ground, often attached to buried grass stems" and on the evidence of the Canberra specimens this fungus is associated strongly with grasses. Even where twig or leaf litter was abundant such substrates bore no *Crinipellis*. Various other areas were searched, but without success. The other sites included habitats similar to the ones above as well as some in which the dominant (sometimes the only) grasses present were weedy, exotic species. The intention was to see if *Crinipellis australis* grew on non-native grasses. The Canberra evidence suggests that the fungus prefers native grasses and in particular seems partial to *Themeda*. In the sites listed above

other native grasses (most commonly *Austrostipa* or one or other of *Austrodanthonia* and *Nothodanthonia*) were often present, but even when *Themeda* was not the dominant grass *Themeda* tussocks would commonly support the majority of sporocarps. In contrast to the other native grasses the *Themeda* tussocks often contain a sizeable proportion of dead material, which presumably provides a good volume of substrate for the fungus.

The specimens cited by Grgurinovic (1997) were collected in the months of March and April and one of the Canberra collections was made in June. These are the cooler, moister autumn months in which one typically expects to see agarics. The bulk of the Canberra collections were gathered in the early summer of 2004/2005. During this period Canberra experienced significant rainfall. The Bureau of Meteorology (2004, 2005) reported that November and December of 2004 were a little warmer and much wetter than average in the ACT. The mean daily minimum temperatures were 9.3°C (November) and 12.0°C (December). The corresponding mean daily maxima were 23.4°C and 26.6°C. Each of these was no more than one degree above average. At Canberra Airport the November rainfall was 85.6 mm over 10 days (well above the November average of 63.4 mm) with most rain falling in the first half of the month, the heaviest fall, of 34.0 mm, being on the 12th of the month. Rainfall was variable across the ACT. The airport's December rainfall was 72.4 mm over 10 days (the average being 52.5 mm over seven days), with nine thunderstorm days (the greatest number of December thunderstorms since 1971). Most of the rain fell between the 5th and the 14th days of the month, with the last two weeks of the month being rather dry. Heavy rain and thunderstorms were quite widespread and most of the ACT weather stations recorded well above average rainfall.

The November/December sightings of *Crinipellis australis* (and in such profusion) suggest that the species is able quickly to exploit good local rain. Such an ability would be of considerable benefit, especially in areas without dense tree or shrub cover, such as grasslands and open woodlands. In such habitats the ground is exposed to the full force of the drying effects of sun or wind. Another characteristic that would help *Crinipellis australis* cope with exposed habitats is that the sporocarps are marcrescent. Several pilei, either collected dry or allowed to dry naturally after collection, gave copious spore prints after rehydration. The ACT receives variable amounts of rain during the warmer months. Often a short period of cool, rainy days is followed by warm to hot and dry days. It is common to see sporocarps of various agarics or members of the Lycoperdaceae appear, develop a little and then abort during the hot days. It is also not too unusual to see aborted sporocarps in exposed areas during autumn, whenever drying winds are severe. It is likely that similar observations could be made in many other areas of south-eastern Australia. In such cases the resources put into sporocarp development have been wasted. With *Crinipellis australis* marcrescence would guard against such a waste of resources and allow the fungus to take advantage of several short rainy periods that are separated by dry periods. The hairy surfaces would presumably help the fungus make the most of any available moisture. They would help trap moisture, for example by acting as condensation points for overnight dew or for fine misting rain. The hairs may also slow down the loss of water from the sporocarp surface. The species therefore seems well-adapted to life in grasslands and open woodlands.

Experiment showed that the hairs of the stipe create a very effective capillary system. Dried specimens, their stipes embedded in a plasticine-like adhesive, were placed in a shallow dish and enough water added to reach those parts of the stipe just above the adhesive surface. Within a few seconds the entire stipe surfaces were wet. That was not the case with some smooth stipes from another species that had been similarly placed. While the *Crinipellis* stipes rehydrated readily in these experiments, the pilei failed to do so, even when left for an hour or more. It appears that the pilei need aerial moisture to rehydrate and cannot make use of a thin, watery film on the soil surface.

Crinipellis australis has been collected from the widely separated Adelaide, Canberra and Sydney areas. In Canberra it has been found in abundance in a variety of grassy habitats

during the summer of 2004/2005. Since that summer I have seen only a small number of *Crinipellis australis* sporocarps in some of the sites listed above. It seems likely that sporocarps could appear at any time of the year, in small numbers after "ordinary" rain but in great abundance after substantial rain spread over a week or so. It is also possible that the species is widespread in (at least) south-eastern mainland Australia and to be found in many grassland and woodland habitats. However the grassland and grassy-woodland habitats of south-eastern mainland Australia are subject to various threats. Many such habitats are in areas ideal for pastoral use or housing development and most have already disappeared or been greatly modified since European settlement. Therefore *Crinipellis australis* may well be widespread but with a very patchy distribution.

A statistician would call the Canberra specimens a biased sample from the *Crinpellis australis* population, for two reasons. First, most of the specimens are the result of a deliberate search for the species during a very short period, in unusual climatic conditions and over a very small area. Second, I ordinarily pay little attention to small, brown agarics, so the fact there was only one previous collection of the species in CANB permits no inference about either the rarity or the spread of the species in the Canberra area. In the absence of further information, there are no grounds for changing the "poorly known" status given to this species by Grgurinovic & Simpson (2001) and the above speculations about the species' distribution and phenology are simply plausible suggestions.

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EDITOR'S NOTE

Readers may have observed that this issue has been numbered volume 26(2-3), and thus we will begin volume 27 with the next issue. The decision to combine issues 2 and 3 into one this year was made in order to catch up with delays getting the journal produced earlier in the year, and was done with the knowledge that we have still published a similar number of pages in volume 26 as have been published in previous volumes, despite these being compressed into two printed issues. Readers can be assured that we will be publishing three separate issues in 2008. In 2008 we intend to submit the journal to Thomson Scientific (who publish the ISI Web of Knowledge) for indexing, and it is hoped that this will lead to a much greater awareness in the international scientific community of papers published in our journal. This will benefit the Society, and the authors who publish in the journal.

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