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QUANTITATION OF CYTOPLASMIC CALCIUM IN GROWING SAPROLEGNIA FERAX HYPHAE USING INDO-1 AND TWO-PHOTON CONFOCAL MICROSCOPY

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Abstract

Ca\(^{2+}\) concentration was determined for the tip, 5 \(\mu\)m and 10 \(\mu\)m intervals between 10 to 80 \(\mu\)m from the tip of growing S. ferax hyphae using the Ca\(^{2+}\)-sensitive fluorochrome Indo-1. Results from this study suggest that there is a statistically significant difference between ratiometric readings, reflecting Ca\(^{2+}\) concentration, 0 to 30 \(\mu\)m from the tip, of two categories—‘slow’ and ‘fast’ growing hyphae. Although ‘slow’ and ‘fast’ growing hyphae exhibited tip-high Ca\(^{2+}\) concentration, slower growing hyphae exhibited a secondary peak 10 \(\mu\)m distal to the tip. The overall Ca\(^{2+}\) concentration, 0–30 \(\mu\)m from the tip, was higher in fast growing hyphae than in slow growing hyphae. The means of the ratiometric readings at 40 \(\mu\)m were not statistically significantly different between slow and fast growing hyphae. The increase in Ca\(^{2+}\) concentration at the base of growing branching hyphae also supports suggestions that there is a relationship between Ca\(^{2+}\) and hyphal growth.


Introduction

Calcium ions (Ca\(^{2+}\)) have been implicated in a wide range of cellular responses including branching, sporulation, tip growth, zoospore motility and cytokinesis (Hyde 1998, Hyde & Heath 1995, Jackson & Hardham 1996, Pitt & Ugalde 1984). There are several lines of evidence to indicate that Ca\(^{2+}\) regulates tip growth including the concentrated expression of stretch-activated Ca\(^{2+}\) channels at the tip (Garrill et al. 1993, Jackson & Heath 1993), Ca\(^{2+}\) regulation of the cytoskeleton and vesicle fusion rates (Jackson & Heath 1993), and the orientation of germinating S. ferax cysts towards a Ca\(^{2+}\)-transporting ionophore, A23187 (Hyde & Heath 1995). During tip growth there is localized transport and fusion of cytoplasm and individual organelles at the tip (McKerracher & Heath 1986). The fine tubules, which make up the vacuolar reticulate structure in the growing tip of S. ferax hyphae, may allow expansion and movement of this organelle to occur while maintaining contact with the large vacuole in the mature regions of hyphae (Allaway et al. 1997). There is some suggestion from selective staining of the vacuolar reticulum with Ca\(^{2+}\) dependent markers that the structure may be involved in the sequestering of Ca\(^{2+}\) (Allaway et al. 1997, Jackson & Heath 1993).

A tip-high gradient of cytoplasmic Ca\(^{2+}\) has been identified by Hyde and Heath (Hyde & Heath 1997) using Fluo-3 and SNARF, and by Garrill et al. (1993) using Indo-1 and the patch-clamp technique. How growth rate correlates with [Ca\(^{2+}\)]i at discrete locations, particularly at the growing tip, has not been established. In this paper, Indo-1 and a highly sensitive two-photon confocal scanning microscope were used to determine the concentration of Ca\(^{2+}\) at discrete locations along the length of slow and fast growing S. ferax hyphae. The advantage of this technique is that it allows minor changes in concentration to be detected by measuring the wavelength shift in Indo-1 emission with changing intracellular Ca\(^{2+}\) concentration. The technique used in this study minimized background and autofluorescence and therefore allowed differences in intracellular Ca\(^{2+}\) concentration to be detected more accurately.

The 405/480 nm emission ratio method for the Ca\(^{2+}\) sensitive ratiometric indicator, Indo-1 was used to measure the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]i). Upon Ca\(^{2+}\) binding, Indo-1 produces changes in fluorescence intensity and a shift in emission from 480 nm to 405 nm (Haugland 1996, Millot et al. 1995). The Indo-1 probe is conjugated with an acetyl-methyl ester (AM) residue to form a neutral Indo-1/AM molecule. In this esterified form Indo-1/AM is able to pass through the cellular membranes while minimising cellular disruption (Millot et al. 1995).
Materials and Methods

Maintenance of *S. ferax* cultures
The method outlined in Allaway *et al.* (1997) was used in the preparation of *Saprolegnia ferax* (Gruith.) Thuret. *Saprolegnia ferax* (H302) was obtained from stock cultures kept in the Research School of Biological Sciences, Canberra. In brief, the cultures were initially grown on GYPS agar (Beakes & Gay 1981) at 23°C in darkness until the plates were confluent. Small agar cubes of culture were sectioned and incubated in GYPS liquid medium (GYPSL) \[5 \text{ g D-glucose, 0.5 g peptone, 0.5 g KH}_2\text{PO}_4, 0.15 \text{ g MgSO}_4\cdot7\text{H}_2\text{O and 0.05 g Yeast extract in 1L dH}_2\text{O}\]. Hyphal segments (approx. 0.2 mm in length) were cut aseptically and incubated in GYPSL in sterile watch glasses. By 18-20 hrs the cultures were 10-15 mm in diameter and ready for Indo-1/AM loading.

**Figure 1.** Intracellular calcium calibration in *S. ferax* (0-602 nM).

The mean ratio of emission increased with increasing \([\text{Ca}^{2+}]_i\). Indications of saturation occurred at 150 nM with limited increase in subsequent ratios. The ratio increase was more pronounced from 0 to 17 nM and 17 nM to 38 nM. From 38 nM to 150 nM the increase in emission ratio was less pronounced. The change in emission ratio from 0 nM to 38 nM was greater than between 38 nM and 225 nM.

**Indo-1/AM loading and specimen mounting**
The cultures were rinsed once with GYPSL and incubated in the dark at room temperature with approximately 200 µl of 10 µg/ml Indo-1 AM ester (Molecular Probes Inc.) in GYPSL (pH5) for 30 min. They were kept submerged, in order to minimise disturbance and possible hyphal damage, then gently washed with fresh GYPSL followed by three partial rinses over 10 min. A coverslip was then gently slid under each culture to lift it. The coverslip was then inverted onto a slide over teflon spacers (Heath 1988) so that hyphae were not squashed. The mounted cultures were then left to recover normal morphology and growth. Using the Leica TCP SPII differential interference contrast facility, only viable hyphae exhibiting growth, the presence of cytoplasmic streaming, characteristic vacuolar reticulum and typical general hyphal morphology (Allaway *et al.* 1997, Rees *et al.* 1994) were measured in this study. Hyphal growth measurements were taken before laser irradiation by using an ocular micrometer. Only growing hyphae were used in this study. Growth was determined by measuring the distance travelled by hyphal tips over five minutes. The growth rates of all hyphae were recorded and the median growth rate (5.75 µm/min.) was used to separate slow growing hyphae (less than or equal to 5.75 µm/min.) and fast growing hyphae (greater than 5.75 µm/min.).

Two independent point measurements were taken for each distance (0-80 µm) along the length of eight randomly chosen hyphae in each of three cultures. The measurements for each distance were used to calculate the mean ratio of emission at 405 and 480 nm for that distance. The Student’s t-test (paired two sample for
means) was used for ratiometric results obtained for different distances from the tip. The null hypothesis was that there was no significant difference between the ratiometric means for fast and slow growing hyphae at 0, 5, 10, 30, 40 or 50 μm from the tip, at a significance level of p = 0.05.

Figure 2. Intracellular calibration of Indo-1.

The intracellular [Ca^{2+}]_{i} was equilibrated with the extracellular [Ca^{2+}]_{i} using calibration buffers. Stable emission at 405 nm and 480 nm respectively along the hypha indicated an even distribution of Ca^{2+}. The intensity of emission increased uniformly along the hyphae with increasing Ca^{2+} calibration buffers. Sequestration of Indo-1 was indicated by random increases in emission along the length of the hyphae.

Figure 3. Emission of Indo-1 in a fast growing hypha.

Binding of Indo-1 to Ca^{2+} results in an emission shift from 480 nm to 405 nm respectively. Emission at 405 nm increased towards the tip indicating increased binding to Indo-1. By 5 μm Indo-1 emission decreased and continued to do so further from the tip. Emission levels reflect level of Ca^{2+} binding. No sequestering of Indo-1 was noted.

Figure 5. Saprolegnia ferax hyphal bud.

Ratiometric measurements (0.16±0.01) at the base (3–5 μm) of the hyphal bud (n = 5) indicated an increase in [Ca^{2+}]_{i} to 38 nM. Based on these limited results it is difficult to determine whether the increase in [Ca^{2+}]_{i} initiates budding or results from budding. The [Ca^{2+}]_{i} calculated at the base of the bud (3–5 μm) is the same as that for fast growing hyphae 5 μm from the tip.

Intracellular Calibration

Calibrated solutions of known Ca^{2+} concentration were obtained in kit form as a range of premixed Ca/EGTA (0 to 10.0 mM with corresponding free Ca^{2+} values ranging from 0 to 3900 nM from BioScientific Pty Ltd (Gymea, Australia). The pH was maintained at 7.2. Intracellular [Ca^{2+}]_{i} was equilibrated with the extracellular [Ca^{2+}]_{i} (Millot et al. 1995). The walls were permeabilised with 0.05% Triton-X (Sigma) in GYPSL for 10 min. (20°C) (Wang & Zhou 1999), washed three times with GYPSL and then incubated in the dark at room temperature with calibration buffer containing 10 μg/ml of Indo-1 for 30 min. The hyphae were mounted on teflon strips as described previously. The mean ratio of emission at 405 and 480 nm was calculated from measurements taken at random distances along the length of five hyphae randomly chosen in each of three cultures.
Measurements of fast growing hyphae (7.2±0.91 μm/min.) indicated higher ratiometric readings than slow growing hyphae (4.6±0.56 μm/min.). Emission ratios correlate directly with $[\text{Ca}^{2+}]_i$. A secondary peak at 10 μm was only observed in slow growing hypha. The significant trough at 30 μm in slow growing hyphae may be due to continued retraction of $\text{Ca}^{2+}$.

**Autofluorescence**

Autofluorescence was determined for each culture by the addition of 20 mM MnCl$_2$ (Sigma Aldrich, Castle Hill, Australia) in calibration buffer or GYPSL as described by Monteith et al. (1997) to quench Indo-1 fluorescence. The background-corrected ratios were calculated by the subtraction of autofluorescence (10-20%) from the mean ratio of emission at 405 and 480 nm. $[\text{Ca}^{2+}]_i$ was determined by using a calibration curve (Figure 1).

**Microscope**

A Leica TCS SP2 MP two photon confocal laser scanning microscope was used to measure Indo-1 emission intensity (Exc. 730 nm, PMT1 Band 385-410 nm, Gain 900V; PMT2 Band 455-495 nm, Gain 900V; Scan mode-xyz; Format 512 × 512; Pinhole[AV] airy; Objective-HCX PL APO 63×/1.20 W CORR). Images were saved as files.

**Results**

**Intracellular calibration of Indo-1**

Intracellular $[\text{Ca}^{2+}]_i$ was equilibrated with the extracellular $[\text{Ca}^{2+}]_i$ using the calibration buffers ($\lambda_1/\lambda_2$). Figure 1 indicates a gradual increase in the emission ratio as concentration increased up to 17 nM. A direct correlation between increasing ratio and increasing $[\text{Ca}^{2+}]_i$ was not evident for concentrations above 150 nM as the saturation level was reached. Only hyphae not showing sequestration of Indo-1 were included (Figure 2).

**Influence of growth rate on the calcium gradient along S. ferax hyphae**

Laser irradiation of hyphae with the two-photon confocal scanning microscope did not appear to cause any deleterious morphological effects or inhibit growth. Viability of the hyphae was checked by differential interference contrast microscopy before and after laser irradiation. Only one fast growing hypha failed to maintain all the indicators of viability, including growth, cytoplasmic streaming, an undisturbed vacuolar reticulum and general hyphal morphology. The hypha was therefore excluded from the data. The images were obtained from 4 irradiating scans, each taking approximately 5 seconds. The mean preimaging growth rate was 5.83±0.22 μm/min. (n = 46). The relationship between calcium gradient and growth rate was investigated by subdividing the population of measured hyphae, using the growth median (5.75 μm/min.), into ‘slow’ and ‘fast’ growing hyphae with mean growth rates of 4.6±0.11 μm/min. (n = 24) and 7.16±0.19 μm/min. (n = 22) respectively. In growing hyphae, ratiometric values increased towards the tip of the hypha (Figure 3). Differences in emission ratio which reflect $[\text{Ca}^{2+}]_i$ were observed for slow and fast growing hyphae. These were statistically significant at 0 μm (t = -6.1, df = 21, p = 5 × 10$^{-5}$), 5 μm (t = -7.5, df = 21, p = 2.4 × 10$^{-7}$), 10 μm (t = -6.7, df = 21, p = 1.4 × 10$^{-6}$) and 30 μm (t = -3.2, df = 21, p = 4 × 10$^{-3}$) from the tip (Figure 4), but not at 40 μm (t = 1.8, df = 17, p = 0.08) or 50 μm (t = 1.3, df = 17, p = 0.2). No differences were detected between slow and fast growing hyphae.
fast growing hyphae at 60 \mu m, 70 \mu m and 80 \mu m distal to the tip. Using Figure 1, [Ca^{2+}]i was calculated for slow and fast mean growth rates (Table 1). Calculated calcium levels for slow growing hyphae ranged from 28 nM at the tip, to a 13 nM plateau 40 \mu m to 80 \mu m distal to the tip. Slow growing hyphae exhibited a secondary peak of 17 nM at 10 \mu m, which was not evident at 10 \mu m in fast growing hyphae (Figure 4). Calculated calcium levels for fast growing hyphae ranged from 125 nM at the tip, to 8 nM, 80 \mu m distal to the tip. In fast growing hyphae, calcium levels decreased from 125 nM at the tip to 38 nM, 5–10 \mu m distal to the tip. A further decline in calcium levels was observed at 20 \mu m (17 nM), 30 \mu m to 60 \mu m (11 nM) and 80 \mu m (8nM).

Emission ratios for hyphal buds 3–5\mu m in length (n = 5) were also measured and the [Ca^{2+}]i calculated to be 38 nM (Figure 5).

Table 1. Calcium concentration estimates for slow and fast growing S. ferax hyphae. In slow growing hyphae (n = 24), [Ca^{2+}]i decreased from 28 nM at the tip to 8 nM at 30 \mu m. A secondary increase in [Ca^{2+}]i at 10 \mu m (17 nM) was noted. From 40 to 80 \mu m there was no change in [Ca^{2+}]i. In fast growing hyphae (n = 22), the [Ca^{2+}]i changed more steeply from 125 nM at the tip to 11 nM at 30 \mu m. There was no secondary increase in [Ca^{2+}]i at 10 \mu m. There was little change in [Ca^{2+}]i beyond 10 \mu m.

<table>
<thead>
<tr>
<th>Location</th>
<th>Slow Growth Rates (4.6±0.56\mu m/min)</th>
<th>Fast Growth Rates (7.16±0.91\mu m/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Ratio</td>
<td>SE</td>
</tr>
<tr>
<td>tip</td>
<td>0.15</td>
<td>0.007</td>
</tr>
<tr>
<td>5\mu m</td>
<td>0.12</td>
<td>0.006</td>
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<tr>
<td>10\mu m</td>
<td>0.13</td>
<td>0.006</td>
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<tr>
<td>20\mu m</td>
<td>0.12</td>
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<tr>
<td>30\mu m</td>
<td>0.1</td>
<td>0.004</td>
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<tr>
<td>40\mu m</td>
<td>0.13</td>
<td>0.003</td>
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<td>50\mu m</td>
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<td>60\mu m</td>
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<td>70\mu m</td>
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<td>80\mu m</td>
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</table>

Discussion

Using Indo-1/AM ester and the two-photon confocal laser scanning microscope it was shown that slow (4.6±0.11 \mu m/min.) and fast (7.16±0.19 \mu m/min.) growing S. ferax hyphae have tip-high [Ca^{2+}]i gradients of around 28 and 125 nM respectively. To allow comparison with the literature ratiometric readings of slow and fast growing hyphae were combined (5.83±0.22 \mu m/min.) and the [Ca^{2+}]i was estimated to be 65 nM (0.17±0.005) at the tip. Using Fluo-3 and SNARF, Hyde & Heath (1997) estimated a tip-high concentration of 76 nM. The results from this study suggest that estimates of [Ca^{2+}]i near the tip (0–30 \mu m) are influenced by growth rate.

There are several lines of evidence that support the theory that the tip-high [Ca^{2+}]i influences growth (Hyde & Heath 1997, Levina et al. 1994). During growth, the Ca^{2+} sensitive filamentous actin cap of an S. ferax hypha undergoes cytoplasmic contraction and strengthening (Jackson & Heath 1993). The concentration of stretch-activated Ca^{2+} channels at the tip is mediated by an actin-dependent system (Garrill et al. 1993, Levina et al. 1994). Similar observations of tip-high [Ca^{2+}]i gradient have been demonstrated in growing pollen tubes, root hairs and S. ferax and Achlya spp. hyphae (Jackson et al. 2001, Obermeyer & Weisenseel 1991, Reiss & Heath 1979, Yuan & Heath 1991). The distribution of mitochondria, endoplasmic reticula and Golgi bodies located approximately 8 \mu m distal to the tip may also contribute to a tip-high [Ca^{2+}]i gradient exhibited by S. ferax.
In this study, hyphal growth ranged between 4 and 8.5 \mu m/min. Subdivision of growth rates at the median into 'slow' and 'fast' growing hyphae made it possible to observe statistically significant differences between emission ratios of slow and fast growing hyphae 0 to 30 \mu m from the tip. In contrast, there was no significant difference in emission ratios at 40 or 50 \mu m from the tip. Similarly, no differences in emission ratios were observed 60, 70 and 80 \mu m from the tip (Figure 4). The higher ratiometric readings of fast growing hyphae (0 to 30 \mu m) suggest a direct correlation between \[Ca^{2+}\] in S. ferax and growth. In S. ferax hyphae \([Ca^{2+}]\) was estimated from the tip (0, 5, 10, 20 and 30 \mu m) to be 28, 13, 17, 13 and 8 nM in slow growing hyphae and 125, 38, 38, 17 and 11 nM in fast growing hyphae (Table 1). The results suggest that \[Ca^{2+}\] gradient increases as growth rate increases. A similar correlation between growth rate and \([Ca^{2+}]\) has been observed by Hyde & Heath (1997).

It is possible that the observed differences in \[Ca^{2+}\] distribution in slow and fast growing hyphae may be due to the retraction of \[Ca^{2+}\] from the vacuolar reticulum at the tip into the central vacuole. The central vacuole is a potential calcium sink (Allaway et al. 1997) located approximately 10–20 \mu m distal to the tip. The continuing retraction of \[Ca^{2+}\] into the central vacuole may account for the irregularity in ratiometric readings 10–40 \mu m from the tip of slow growing hyphae. Whether \([Ca^{2+}]\) influences growth or vice versa is not clear from these results. A larger sample size may provide a clearer indication of the relationship between growth and \([Ca^{2+}]\).

Unlike S. ferax, \([Ca^{2+}]\) in Neurospora appeared to peak 10 \mu m from the tip using non-ratiometric \[Ca^{2+}\] dye, Fluo-3 (Jackson & Heath 1993). A similar observation was made using ratioed Fluo-3 and SNARF-1 in Neurospora (Levina et al. 1994). The potential sources of \[Ca^{2+}\] in Saprolegnia and Neurospora could provide a possible explanation for these differences. In Saprolegnia the \[Ca^{2+}\] source is more likely to be from the tip-localized stretch-activated \[Ca^{2+}\] channels (Garrill et al. 1993), whereas in Neurospora, the subapical stores of \[Ca^{2+}\] may contribute to the peak at around 10 \mu m (Levina et al. 1994).

The observed decrease in \[Ca^{2+}\] 5 \mu m distal to the tip may be due to the lack of distinction in emission readings of the vacuolar reticulum, the central vacuole, and the surrounding cytoplasm. Subcellular measurements of plants have shown that the central vacuole \([Ca^{2+}]\) levels were three to four times higher than the surrounding cytoplasm (Gilroy et al. 1993). The \[Ca^{2+}\] gradient declined gradually from the tip and plateaued 40–70 \mu m distally. This is unlike Hyde & Heath’s (1997) observation of a steep decline in \[Ca^{2+}\] at 40 \mu m. The central vacuole extending throughout this region, is most likely complemented by increasing numbers of nuclei (Yuan & Heath 1991).

In developing branches of the hyphae there were indications of elevated levels of \[Ca^{2+}\] (38 nM) at the base of 3–5 \mu m long hyphal buds (Figure 5). The presence of ‘hotspots’ or localized elevated readings of \[Ca^{2+}\] early in branching may act as a special cue or morphogen and define the axis of polarity (Hyde & Heath 1995, Jackson et al. 2001). Evidence supporting \[Ca^{2+}\] as a morphogen in the growing tip include its regulation of actin, stretch-activated \[Ca^{2+}\] channels, cytoskeletal changes and vesicle fusion (Garrill et al. 1993, Jackson & Heath 1993). There is, however, inconclusive evidence to indicate that \[Ca^{2+}\] is essential for the earlier stages of branch emergence (Jackson et al. 2001). It is not clear from these preliminary observations whether the elevated levels of \[Ca^{2+}\] trigger budding or whether another factor initiates \[Ca^{2+}\] accumulation.

It has been observed with Fluo-3 and SNARF that subapically \([Ca^{2+}]\) was higher in the periphery of S. ferax hyphae than in the centre (Hyde & Heath 1997). The \[Ca^{2+}\] sequestering mitochondria located along the wall (Yuan & Heath 1991) may contribute to differential spatial distribution of \([Ca^{2+}]\) from the middle of the hypha to the periphery. Results with Indo-1 were not able to demonstrate changes in the distribution of \[Ca^{2+}\] along the width of S. ferax hyphae in subapical locations. \([Ca^{2+}]\) was, however, not measured beyond 80 \mu m where mitochondria localization was noted (Yuan & Heath 1991). The central vacuole, 80 to 100 \mu m distal to the tip, takes up a large proportion of the hypha. The restriction of the mitochondria to the periphery suggests the low \([Ca^{2+}]\) exhibited in this area may reflect the \([Ca^{2+}]\) present in the central vacuole alone.
Evidence from this study suggests that the growing hyphae of *S. ferax* have a tip-high gradient of Ca\(^{2+}\). There appears to be a correlation between growth rate and Ca\(^{2+}\) gradient, particularly 0 to 30 \(\mu\)m from the tip. The increase in ratiometric reading 10 \(\mu\)m from the tip in slow growing hyphae may reflect physiological and spatial changes in the Ca\(^{2+}\) gradient during growth.

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**References**


THE ULTRASTRUCTURE OF HYDROGENOSOMES IN THIN SECTIONS AND IN FREEZE FRACTURE REPLICA FROM THE ANAEROBIC CHYTRID FUNGUS CAECOMYCES SP.

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Abstract

Hydrogenosomes from the obligately anaerobic rumen fungus Caecomyces equi were examined in thin sections and freeze fracture replicates with the transmission electron microscope. These organelles were bounded by a single unit membrane and appeared to contain granular material inside. Sometimes a single unit internal membrane was also present. The dumbbell shape of hydrogenosomes seen in some thin sections and freeze fracture replicates suggests the possibility that these organelles divide by binary fission. There is no evidence that there is an associated organelle genome.

Introduction

The presence of the hydrogenosomes together with the absence of mitochondria have been observed in all isolates of obligately anaerobic rumen fungi which have been studied with the electron microscope (Munn 1994, Munn et al. 1988, Trinci et al. 1994). Hydrogenosomes were first discovered in obligately anaerobic protozoa which lack mitochondria (Muller 1988). They are membrane-bound organelles that produce ATP and molecular hydrogen (Muller 1993).

The five known genera of rumen fungi have been placed by Li et al. (1993) in the order Neocallimasticales (Chytridiomycetes). The fungi in this order are obligately anaerobic, lack the oxidative type of metabolism found in most fungi and rely on fermentation of carbohydrates for the production of ATP (Trinci et al. 1994). Yarlett et al. (1986) and Marvin-Sikkema et al. (1993a, b) have studied some of the enzyme systems within hydrogenosomes isolated from only one of the five genera, Neocallimastix. In fact, with the exception of morphological and nutritional studies, most of the research on rumen fungi has been done with this one genus. The other four genera await further characterization at the molecular level.

The role of the hydrogenosome in the energy metabolism and production of ATP in obligately anaerobic fungi and the possible relationship of hydrogenosomes to mitochondria have been reviewed by Marvin-Sikkema et al. (1994), Trinci et al. (1994), Embley et al. (2002) and Voncken et al. (2002).


We examined both thin sections and freeze fracture replicas of surfaces of sections through cells of one species from the rumen fungi, Caecomyces equi Gold, Heath & Bauchop, with the transmission electron microscope in order to study the structure of the membranes surrounding hydrogenosomes, the internal structure of
hydrogenosomes and other ultrastructural features. The freeze fracture technique had been used only once previously to study ultrastructure of the protoplasm in general or hydrogenosomes in specific in the rumen fungi (Benchimol et al. 1997). However, Benchimol et al. (1997) only published one photograph and that was of a hydrogenosome from Neocallimastix. Data from freeze fracture replicas should give us more insight into the structure of membranes surrounding and within the hydrogenosome in the rumen fungi. The number of membranes surrounding the hydrogenosome is important for understanding the evolutionary origin of this organelle and its possible relationship to the mitochondrion (Benchimol et al. 1997, van der Giezen et al. 1997).

Materials and Methods

NM1, NJ11 and NJ12 were isolated from the rumen of sheep in Australia using the procedure described by Phillips & Gordon (1988, 1989) and were maintained at 39°C since isolation by transfer to fresh anaerobic growth medium every 4 to 5 days. These isolates of obligately anaerobic fungi resembled Sphaeromonas communis Orpin in morphology (Orpin 1976), but are now classified as Caecomyces communis Gold, Heath & Bauchop (Gold et al. 1988).

The fungi were grown anaerobically in serum bottles with butyl rubber stoppers and aluminium crimp seals at 39°C in liquid, pre-reduced basal medium 10 plus either glucose at a concentration of 5 g per litre or finely milled wheat straw (passing a 1 mm screen) at a concentration of 10 g per litre. No agar was added to the medium. The complete composition of pre-reduced basal medium 10 and the method for medium preparation are described by Phillips & Gordon (1988, 1989).

The fungal cells from NM1, NJ11 and NJ12 were fixed in anaerobic cultures by slowly adding 50% glutaraldehyde solution from a syringe through the stopper into the culture bottle to give a final concentration of 3% (v/v) in the growth medium. For cells used to make thin sections the fixation time was 3 hours at room temperature. After washing three times with 0.1 M sodium cacodylate buffer at pH 7.4, the cells were post-fixed for two hours with 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer at pH 7.4. After post-fixation the cells were washed again three times with cacodylate buffer, dehydrated through a series of graded ethanol solutions to 100% ethanol either with or without 100% propylene oxide in the last step prior to infiltration and then were infiltrated and embedded in low viscosity epoxy resin or araldite resin. Ultra thin sections were cut using a diamond knife fitted to a Reichert-Jung Ultracut C ultramicrotome. The sections were then stained using stabilised solutions of uranyl acetate and lead citrate and placed on a grid prior to examination with a Philips EM 300 transmission electron microscope.

For cells of NM1 used for freeze fracture, the fixation time with 3% glutaraldehyde was 1 hour at room temperature. After fixation the cells were infiltrated with 50% glycerol (v/v) for 1 hour and then concentrated by low speed centrifugation. The cells were frozen as quickly as possible by plunging them into liquid nitrogen cooled freon F22 and then were transferred into liquid nitrogen. The cells were fractured using a Balzers high vacuum freeze fracture unit (Model BAE 300) while the specimen was maintained at -100°C and the cutting knife at -196°C. The surface of the fractured cells was shadowed with platinum-carbon to give a thickness of 20 nm and then coated with a carbon layer for backing with a thickness also of 20 nm. The remains of the living tissue were removed from the replica by digestion overnight in 50% chromic acid at room temperature. The replica was then washed twice with distilled water, placed on a grid and examined with the electron microscope.

Results

Examination of thin sections of sporangia and rhizoids of Caecomyces equi NM1, NJ11 and NJ12 revealed that the most conspicuous organelles within the cell were nuclei, hydrogenosomes, crystal bodies, vacuoles, and flagella (formed within vacuoles in the sporangia). Other structures seen within the cells include ribosomes (in packets in zoospores developing within the sporangium and free within the cytoplasm of other cells), microtubules (in flagella and forming intra-nuclear spindles within dividing nuclei), rarely endoplasmic reticulum, cell membrane, vacuolar membrane and cell wall. The cell wall often had a fibrillar coating on the external surface, which probably is involved in attachment to substrates. We used nuclear structure as an indication of quality of fixation and staining. The nucleus was bounded by a double membrane with nuclear pores as in all eukaryotic cells (Fig. 1). Therefore the procedure for fixation and staining appeared to give good results.
Nuclei, hydrogenosomes and the cell walls were easily observed in replicas of sections made through cells of *Caecomyces* Theodorou 1988, Yarlett 1986). The observations of the structure of hydrogenosomes in *NM1* et al. 1987, Munn 1988, Webb & 1993a, b, Munn 1994, Munn Marvin-Sikkema et al. (1988) and the isolates of other genera of obligately anaerobic rumen fungi (Gaillard & Citron 1989, et al. authors (Gaillard & Citron 1989, Gold et al. et al. et al. et al. 1992, Marvin-Sikkema et al. 1993a, b, Munn 1994, Munn et al. 1981, Munn et al. 1987, Munn et al. 1988, Webb & Theodorou 1988, Yarlett et al. 1986). The observations of the structure of hydrogenosomes in *Caecomyces* NM1 using the freeze fracture technique confirm that the hydrogenosome in the fungi in the Order Neocallimasticales is bounded by a single unit membrane as seen in thin sections in this study as well as in other studies by various authors (Gaillard & Citron 1989, Gold et al. 1988, Heath et al. 1983, 1985, Li et al. 1990, Li et al. 1991, Marvin-Sikkema et al. 1992, Marvin-Sikkema et al. 1993a, b, Munn 1994, Munn et al. 1981, Munn et al. 1988, Webb & Theodorou 1988, Yarlett et al. 1986). The internal membrane can be seen in several electron micrographs of thin sections of hydrogenosomes from *Neocallimastix* (Marvin-Sikkema et al. 1992, Marvin-Sikkema et al. 1993a, Munn 1994). In our study, one inner membrane appears to touch the outer membrane at a right angle. The resolution in our sections was not good enough to determine precisely the continuity of the lipid bi-layers at this point, but all membranes consist of one unit membrane, approximately the same thickness as the cell and vacuolar membranes. Furthermore, the exact origin of the inner membrane cannot be determined by the methods used here. Because it does not completely cross the internal volume of the hydrogenosome, it may be formed independently of the outer membrane. The inner membranes within the hydrogenosomes were always a single unit membrane. The internal matrix of the hydrosome appears to be granular which is consistent with the fact that it is filled with enzymes (Benchimol et al. 1997, Marvin-Sikkema et al. 1994, Yarlett et al. 1986).

**Discussion**

In general the ultrastructure of *Caecomyces equi* isolates NM1, NJ11 and NJ12 appeared similar to that of the isolates of this genus which have been previously studied by Gaillard & Citron (1989), Gold et al. (1988) and Munn et al. (1988) and the isolates of other genera of obligately anaerobic rumen fungi (Gaillard & Citron 1989, Gaillard-Martine et al. 1992, Heath & Bauchop 1985, Li et al. 1990, Li et al. 1991, Marvin-Sikkema et al. 1992, Marvin-Sikkema et al. 1993a, b, Munn 1994, Munn et al. 1981, Munn et al. 1987, Munn et al. 1988, Webb & Theodorou 1988, Yarlett et al. 1986). The observations of the structure of hydrogenosomes in *Caecomyces* NM1 using the freeze fracture technique confirm that the hydrogenosome in the fungi in the Order Neocallimasticales is bounded by a single unit membrane as seen in thin sections in this study as well as in other studies by various authors (Gaillard & Citron 1989, Gold et al. 1988, Heath et al. 1983, 1985, Li et al. 1990, Li et al. 1991, Marvin-Sikkema et al. 1992, Marvin-Sikkema et al. 1993, Munn 1994, Munn et al. 1981, Munn et al. 1988, Webb & Theodorou 1988, Yarlett et al. 1986). The internal membrane can be seen in several electron micrographs of thin sections of hydrogenosomes from *Neocallimastix* (Marvin-Sikkema et al. 1992, Marvin-Sikkema et al. 1993a, Munn 1994). In our study, one inner membrane appears to touch the outer membrane at a right angle. The resolution in our sections was not good enough to determine precisely the continuity of the lipid bi-layers at this point, but all membranes consist of one unit membrane, approximately the same thickness as the cell and vacuolar membranes. Furthermore, the exact origin of the inner membrane cannot be determined by the methods used here. Because it does not completely cross the internal volume of the hydrogenosome, it may be formed independently of the outer membrane. The inner membranes within the hydrogenosomes were always a single unit membrane. The internal matrix of the hydrosome appears to be granular which is consistent with the fact that it is filled with enzymes (Benchimol et al. 1997, Marvin-Sikkema et al. 1994, Yarlett et al. 1986).

The dumbbell shape of some hydrogenosomes in thin sections and freeze fracture replicas suggests the possibility that these organelles divide by binary fission or that this organelle has plasticity in shape. This has been proposed previously by Munn (1994) and by Benchimol et al. (1997).

Benchimol et al. (1997) and van der Giezen et al. (1997) both found two closely apposed membranes surrounding the hydrogenosomes and, in some preparations, double membranes within the hydrogenosomes from *Neocallimastix*. There was no inter-membrane space between either the two outer or the two internal membranes. Benchimol (1997) observed two membranes surrounding the hydrogenosome in freeze-fracture replicas as well. Benchimol et al. (1997) explains that their discovery of double membranes in the hydrogenosome is due to better fixation, staining and sectioning methods and to higher magnification. However, in our study with *Caecomyces* two membranes were not seen in either thin sections or freeze-fracture replicas. This raises the possibility that the outer and inner membranes can be either double or single in the rumen fungi.
Hydrogenosomes have also been found in some obligately anaerobic protozoa from a number of unrelated evolutionary lines: for example, parasitic parabasalian flagellates (Order Trichomonadida), rumen and intestinal ciliates (Orders Entodiniumorphida and Trichostomatida), some free living ciliates (Order Heterotrichida, Order Odontostomatida and several other orders) and in at least one amoeboflagellate (Genus Psalteriromonas, Heterolobosea) (Muller 1993). A number of other species of obligately anaerobic protozoa lack both hydrogenosomes and mitochondria (Muller 1993). In the trichomonads the hydrogenosome is bounded by a double unit membrane as seen in thin sections and freeze fracture replicates (Honigberg et al. 1984). In the ciliates, the hydrogenosome can be bounded by either a single or a double unit membrane (Muller 1993). This and other data have lead Muller (1993) to speculate that the hydrogenosome has evolved independently many times. Currently mitochondria and hydrogenosomes are considered to be two forms of the same organelle (Embley et al. 2002). However, it is necessary to make sure that the fungal hydrogenosome does not have a second membrane that is destroyed by conventional processing for observation in the transmission electron microscope. Our freeze fracture data shows that this is not the case and the fungal hydrogenosome is surrounded by one unit membrane. More investigation is needed to resolve this issue.

Electron microscope studies have never provided evidence for an organelle genome in the hydrogenosome. Furthermore, van der Giezen et al. (1997) were unable to detect any DNA in hydrogenosomal fractions from Neocallimastix in contrast to mitochondria from aerobic fungi.

Both obligately anaerobic rumen fungi (Trinci et al. 1994) and obligately anaerobic protozoa (Muller 1988) can synthesize ATP by fermentation of glucose to either lactic acid or ethanol. Enzymes that catalyze the chemical conversion of glucose to lactic acid or ethanol are normally found in the cytosol. The enzymes in the hydrogenosome provide an alternative pathway for fermentation of glucose with the production of hydrogen gas (Marvin-Sikkema et al. 1994, Trinci et al. 1994). However, several isolates of fungi in the genus Blastocladia, which is related to the rumen fungi (Gleason & Gordon 1989), and many genera of protozoa related to the orders mentioned above (Muller 1993) are capable of growth under anaerobic conditions without hydrogenosomes because they have all necessary enzymes in the cytosol. Why then is the hydrogenosome found in evolutionarily unrelated groups of obligately anaerobic eukaryotic micro-organisms? Voncken et al. (2002) suggest multiple origins of hydrogenosomes. The hydrogenosome forms a compartment separate from the cytosol where different chemical reactions can take place. Marvin-Sikkema et al. (1994) and Yarlett et al. (1986) have isolated a number of enzymes from the hydrogenosome that catalyze reactions leading to the production of hydrogen gas. The function of the outer membrane of the hydrogenosome is not understood, although Marvin-Sikkema et al. (1994) propose that an ATPase is involved in maintenance of a protomotive force across the membrane. They suggest that the protomotive force may function in transport of metabolites both into and out of the hydrogenosome and possibly transport of proteins into the hydrogenosome. Also, they suggest that proton pumping from the matrix of the hydrogenosome into the cytosol results in the generation of a pH gradient across the hydrogenosomal membrane. This could provide an optimal pH in the matrix to allow efficient enzymatic conversion of the metabolites that have been transported inside. According to Voncken et al. (2002) a mitochondrial-type ADP/ATP carrier has been identified in the hydrogenosomes of Neocallimastix. This provides more evidence that the hydrogenosomes of rumen fungi evolved from fungal mitochondria (Voncken et al. 2002).

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References


Fig. 1. Thin section of the nucleus showing the double membrane with nuclear pores (NJ11). Fig. 2. Thin section of a hydrogenosome (H), membrane around hydrogenosome, vacuole (V) and vacuolar membrane (NJ11). Fig. 3. Thin sections of hydrogenosomes, one of which has an internal membrane (NJ12). Fig. 4. Freeze fracture replica of the nucleus showing the outer surfaces of the inner and outer membranes (NM1). Fig. 5. Freeze fracture replica of the nucleus with nuclear pores (NM1). Fig. 6. Freeze fracture replica of a hydrogenosome showing outer and inner surfaces of the membrane (NM1).

Fig. 7. Freeze fracture replica of a hydrogenosome showing inner surface of the membrane (NM1). Fig. 8. Freeze fracture replica of a spherical hydrogenosome showing the outer surface of the membrane and granules in the centre (NM1). Fig. 9. Freeze fracture replica of a dumbbell shaped hydrogenosome showing the inner and outer surfaces of the membrane and granules in the centre (NM1). Fig. 10. Freeze fracture replica of a hydrogenosome showing a groove on the outer surface (NM1).

Scale bars: Fig. 1, 230 nm; Fig. 2, 68 nm; Fig. 3, 120 nm; Fig. 4, 260 nm; Fig. 5, 68 nm; Fig. 6, 89 nm; Fig. 7, 154 nm; Fig. 8, 138 nm; Fig. 9, 159 nm; Fig. 10, 88 nm.


Despite our knowledge of the unique flora and fauna of Australia, much less is known about fungal diversity. Eighty-six species of chytrid fungi (Chytridiomycota) have been reported from soils of Australia. The primary purpose of this study was to assess the biodiversity of chytrids in four distinct vegetation types in central eastern New South Wales: subtropical rainforest, wet sclerophyll forest, dry sclerophyll forest and open heath. Attention was focused especially on newly observed species, new records of taxa in Australia, and morphological variation of known taxa. A second objective was to assess species richness and diversity of chytrids within the four habitats. Water cultures of 227 soil samples from 14 collection sites were baited with cellulose, chitin, keratin, and pollen substrates. The substrates were examined microscopically for the presence of chytrids, and 38 taxa were observed. Evaluation of species diversity among the major collection sites used a presence or absence recording technique, and indicated that the greatest number of species occurred in dry sclerophyll forest, while the least number of species occurred in the open heath habitat. Across all habitats studied, a few chytrid species were common while most were scarce to rare. Many of the 17 species recorded for the first time in Australia also are considered to be pandemic in distribution. Eight taxa were observed for the first time and were assigned provisional generic affiliation, and may be endemic to Australia. This study serves as a baseline for evaluation of chytrid biodiversity and distribution in additional natural and disturbed habitats of Australia.
Jeffrey & Willoughby (1964) studied the distribution of *Allomyces* (Blastocladiales) from a variety of soil groups, and concluded that the taxon could be considered a valid soil inhabitant. Willoughby (1965) investigated soils primarily from Victoria for members of the Chytridiales, for comparison with distribution of chytrids from America and Europe. Using cellulose, chitin and keratin as baits, he obtained 34 species, and found evidence of chytrid distribution patterns related to soil conditions such as type of soil, pH, and cultivation/fertilization regimes. Karling (1988) amplified Willoughby's (1965) inventory by examining 49 soil samples from 13 collection sites across Australia, and identified 15 species of Chytridiales and Blastocladiales.

The purpose of this study was to begin to characterize the chytrid biodiversity in soils of Australia within a structured and defined collecting scheme using an established protocol (Letcher & Powell 2001, 2002a) of selective baiting with a variety of substrates to obtain chytrids. We examined numerous samples of soil from four distinct plant communities—subtropical rainforest, wet sclerophyll forest, dry sclerophyll forest and open heath—and evaluated the diversity of chytrids in the four habitats studied.

### Materials and Methods

Between November 2002 and February 2003, 227 soil samples were obtained from 14 collection sites among four distinct vegetation habitats: dry sclerophyll forest, wet sclerophyll forest, subtropical rainforest and open heath (Table 1). Soils were collected within National Parks under New South Wales National Parks (NP) and Wildlife Service Permit # A3392, or collected on private land with permission of the owners. Collection of soil samples and preparation of enrichment cultures were as previously described (Letcher & Powell 2001, 2002a). Global Positioning System (GPS) coordinates were obtained with a Garmin GPS 12 NAVAID. Chytrids were observed within the soils using a selective enrichment technique (Letcher & Powell 2001). Because soil chytrids exhibit a high degree of substrate specificity (Sparrow 1960), four substrates were added to cultures of each soil sample: cellulose (onion scale epidermis), chitin (purified shrimp exoskeleton), keratin (purified snake skin) and sterile gymnosperm pollen (*Pinus radiata* or *P. strobus*). Enrichment cultures were incubated at 20°C. Substrates were examined microscopically at 5–8 days and again at 14–20 days for the presence of chytrids. For each observational time, presence or absence of individual species in enrichment cultures was recorded.

Species diversity (the relative commonness and rarity of species, Morin 1999), as well as the number of species per site, was evaluated for all collection sites. Species diversity among four distinct habitats was investigated, from four collection sites where the sample size (n = 20–28 samples per collection) and repeat collections (n = 2) were sufficient to reliably indicate this ecological parameter (Letcher & Powell 2001). Each of the four collection sites represented one of the habitats studied.

<table>
<thead>
<tr>
<th>Collection sites</th>
<th>Vegetation type</th>
<th># Samples</th>
<th>GPS coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ourimbah, Palm Grove</td>
<td>subtropical rainforest</td>
<td>48</td>
<td>S 33° 18.356'E 151° 17.093'</td>
</tr>
<tr>
<td>2. Windsor, Valley Centre for</td>
<td>wet sclerophyll</td>
<td>40</td>
<td>S 33° 24.825'E 150° 54.833'</td>
</tr>
<tr>
<td>Environmental Education and</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Research</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Track</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Ku-ring-gai Chase NP, the</td>
<td>dry sclerophyll</td>
<td>45</td>
<td>S 33° 39.837'E 151° 15.264'</td>
</tr>
<tr>
<td>Duckholes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Garigal NP, Deep Creek Reserve</td>
<td>dry sclerophyll</td>
<td>8</td>
<td>S 33° 42.400'E 151° 16.460'</td>
</tr>
<tr>
<td>6. Warringah Council, Wheeler</td>
<td>dry sclerophyll</td>
<td>6</td>
<td>S 33° 44.193'E 151° 17.752'</td>
</tr>
<tr>
<td>Creek</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Royal NP, Hacking River</td>
<td>wet sclerophyll</td>
<td>9</td>
<td>S 34° 09.185'E 151° 01.737'</td>
</tr>
<tr>
<td>8. Morton NP, Fitzroy Falls</td>
<td>dry sclerophyll</td>
<td>6</td>
<td>S 34° 38.798'E 150° 28.941'</td>
</tr>
<tr>
<td>9. Kangaroo Valley, Cambewarra</td>
<td>dry sclerophyll</td>
<td>2</td>
<td>S 34° 47.993'E 150° 34.637'</td>
</tr>
<tr>
<td>Lookout</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Morton NP, Mt Bushwalker</td>
<td>open heath</td>
<td>8</td>
<td>S 35° 14.459'E 150° 18.768'</td>
</tr>
<tr>
<td>11. Morton NP, Porters Creek</td>
<td>dry sclerophyll</td>
<td>2</td>
<td>S 35° 15.501'E 150° 22.330'</td>
</tr>
<tr>
<td>12. Morton NP, Little Forest</td>
<td>dry sclerophyll</td>
<td>4</td>
<td>S 35° 15.888'E 150° 21.343'</td>
</tr>
<tr>
<td>Plateau</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. Murramarang NP, Cullendulla</td>
<td>wet sclerophyll</td>
<td>3</td>
<td>S 35° 40.109'E 150° 17.967'</td>
</tr>
</tbody>
</table>
Results

From 227 soil samples from 14 collection sites (Table 1), 38 species were identified. These are listed and described below. Eight of the taxa may represent undescribed chytrids (Allomyces sp., Blyttioniomyces sp., Chytriomyces sp., Cladochytrium sp., Lacustromyces sp., Powellomyces sp., Rhizophlyctis sp. #1 and Rhizophlyctis sp. #2), and those taxa have been provisionally assigned generic affiliation based on available data. Seventeen additional taxa are new records in Australia of previously described chytrids. Following the name of each observed species, the reference to its description and the figure number of an illustration of the Australian isolate are given, and the notation ‘New record’ is included for those described taxa that have not been previously noted in Australian surveys. Notations of the substrate/s on which the chytrid was isolated (c-cellulose, ch-chitin, k-keratin, p-pollen) and the site/s where each species was collected (location numbers from Table 1) are indicated within brackets.

Taxa identified

**Allomyces sp.** Fig. 1. [ch, 5]. This chytrid was found in a single location, in depauperate sandy soil and in association with the vascular plant *Casuarina cunninghamiana*. The sexual stage has not been observed with material from enrichment cultures or with a pure culture isolate of this fungus.

**Diplophlyctis sarcoptoides** Petersen (1903). New record. Fig. 2. [c, 4, 12]. Morphology of this chytrid agreed well with the original description; however, its occurrence on cellulose rather than chitin was unusual. Sporangia on cellulose were smaller than those of the type species, being 12–20 µm broad x 12–16 µm tall. Branched rhizoids extended from a clearly defined and visible spherical apophysis. Zoospores were discharged as a mass. Resting spores that were smaller than the sporangia were observed in older cultures.

**Blyttioniomyces sp.** Fig. 3. [p, 3, 8, 10, 11]. This organism was reported previously from Virginia, USA, soils as *Phlyctochytrium sp. #2* (Letcher & Powell 2001). Immature sporangia were golden in colour and had a terminal apiculus. Mature, spherical (18–26 µm in diameter) to subspherical sporangia (16–30 µm broad x 12–18 µm tall) developed 3–7 prominent, lateral cone-shaped discharge tubes, and the sporangial wall was covered with orange-brown, irregularly shaped plaques. Within the substrate, a few stout rhizoids arose from a small (6–8 µm in diameter) spherical apophysis. From one or more discharge tubes, zoospores were discharged singly. They remained quiescent just external to the discharge pore for a few seconds before swimming away. Resting spores were not observed. Presence of the apical apiculus, lateral discharge pores, and an apophysis favour the provisional generic placement of this organism.

**Chytriomyces rhiophydil** Karling (1948). New record. Fig. 4. [2]. This chytrid was parasitic on both *Rhizophydium pollinis-pini* and *R. sphaerotheca*. Aggregations of elongate, 8–12 µm broad x 12–24 µm tall sporangia developed on the substrate. Zoospore discharge was abrupt and explosive and, following sporangial dehiscence, the zoospores remained quiescent for a few moments before dispersal. Resting spores were not observed.

**Chytriomyces annulatus** Dogma (1969). New record. Figs 5–7. [ch, p, 2, 3, 4, 5, 10, 11, 12, 13]. This organism presents a remarkable picture of distribution, morphology, and morphological plasticity. First observed in Michigan, USA (Dogma 1969), it has since been reported from several eastern USA states, Canada, and Poland (Letcher & Powell 2002b). This chytrid is readily identified on the basis of its pyriform-shaped sporangium, proximal collar-like annulations that ornament the sporangial wall, and an extramatrical rhizoidal stalk (fig. 5). The sporangial annulations also are apparent on empty sporangia. The Australian material also contained sporangia that were either clavate (fig. 6) or obovoid (fig. 7) and that were sessile upon the substrate. Additionally, the characteristic and diagnostic annulations were not observed with mature and undischarged obovoid sporangia, and only upon observation of discharged sporangia of the obovoid morphology were the diagnostic annulations revealed.

**Chytriomyces hyalinus** Karling (1945). Fig. 8. [ch, k, 1, 2, 3, 4, 8, 11, 12]. The material from Australian soils agreed well with the type material. This chytrid was encountered frequently, reinforcing its proposed cosmopolitan distribution and frequency (Letcher & Powell 2002b).

**Chytriomyces poulatus** Willoughby & Townley (1961). Fig. 9. [p, 1, 4]. Features of this chytrid corresponded well with the type description. Sporangia mostly were ovoid, 12–20 µm broad x 18–35 µm tall, and were ornamented with characteristic overlapping cupules of wall material. At zoospore discharge, the
operculum separated partially from the sporangial wall as the first few zoospores were ejected. Those zoospores remained quiescent at the exit orifice for a few minutes before slowly swimming away. The remaining zoospores began to swim in the sporangium and eventually most escaped and dispersed. Sometimes, however, one or two zoospores remained within, germinated, and proliferated within the sporangium.

It is interesting that the majority of specimens examined by Willoughby (1965) from Australian soils lacked the distinctive cupule surface ornamentation, and that many were irregular in shape as well as multioperculate. Absence of overlapping cupules has been reported by Willoughby (1965), Sparrow (1968), Booth (1971a, b), and Booth & Barrett (1971). Such morphological variation was not observed in material from New South Wales soils in this study. Longcore (1992) described Chytrium angularis, a chytrid morphologically similar to C. poculatus but lacking any wall ornamentation, and which in pure culture did not produce cupules of overlapping wall material. Sporangia also were irregularly gibbose and often in clusters, thus making the two species distinguishable.

**Chytriomyces clade #1.** Figs 33, 34. [c, 2]. This chytrid was observed on cellulose bait with several soil samples from two collection sites. Sporangia were spherical (20–25 μm in diameter), subspherical, or irregularly shaped (20–32 μm broad × 30–45 μm tall). Isodiametric rhizoids, up to 6 μm in diameter, originated at 2–6 points on the sporangium, extended to 200 μm or more in length, and were sparsely branched. Upon discharge, numerous small (2–3 μm diameter) spherical zoospores emerged in a hyaline vesicle from a uniform diameter (6–8 μm) discharge tube 6–40 μm in length, and formed a quiescent cluster at the orifice prior to dispersal. Resting spores were not observed. Ultrastructural studies of the zoospore (unpublished) indicate this chytrid has a Group I-type zoospore (Barr 1980) that is found in all members of the Chytriomyces clade (James et al. 2000) thus far examined; however, its phylogenetic position is yet to be resolved via molecular analysis.

**Chytriomyces clade #2 (‘Miller’s Dentate’).** (Miller 1968). Fig. 19. New record. [ch, p, 1, 4, 6]. The spherical (15–35 μm diameter) ornamented sporangia and tubular interbiotic or endobiotic rhizoid conformed to Miller’s (1968) description. Zoospores were discharged into an exogenous vesicle where they swarmed prior to dispersal into the environment. Molecular analysis (unpublished) places this chytrid in the Chytriomyces clade (James et al. 2000), but zoospore ultrastructure remains to be examined.

This organism is probably a member of a species complex (Miller 1968) that may include Phlyctochytrium aureliae and P. mucronatum. It lacks an apophysis, which is characteristic of Phlyctochytrium, but the dentate sporangial ornamentation is similar to that exhibited by P. aureliae and P. mucronatum.

**Chytriomyces sp.** Figs 10–12. [p, 1]. This operculate chytrid was abundant on pollen bait, but occurred only in a single soil sample. The subspherical (12–16 μm diameter) ornamented sporangia and tubular interbiotic or endobiotic rhizoid conformed to the type material, it colonized only the cellulose bait. Zoospores were discharged into an exogenous vesicle, where they remained quiescent for a few moments before actively dispersing and swimming away.

This fungus resembles Chytriomyces sp. described by Sparrow (1968), a multi-lobed, operculate chytrid that he considered to be allied to C. poculatus. Because so little of the development and life cycle of this organism has been observed, it is not assigned a specific epithet, but rather it is being placed provisionally in Chytriomyces on the basis of the operculate sporangium and epibiotic resting spore.

**Chytriomyces spinosus** Fay (1947). Fig. 13. New record. [c, 6, 10]. This distinctive chytrid was observed in soil from both dry sclerophyll forest and open heath. Consistent with the type material, it colonized only the cellulose bait. Zoospores were discharged into an exogenous vesicle, where they remained quiescent for a few moments before actively dispersing and swimming away.

**Chytriomyces willoughbyi** Karling (1968). Fig. 14. New record. [2, 4, 8]. This parasitic chytrid was observed on Rhizophyllum spherotheca and R. globosum. The operculate sporangium partially collapsed after passive discharge of zoospores.

**Cladochytrium sp.** Fig. 15. [c, 7]. This organism was observed on cellulose in two soil samples from a single site. The intramatrical thallus consisted of fine, highly branched rhizomycelium with one- or two-celled narrowly
elliptical turbinate organs, 7–10 µm wide × 15–18 µm long, interspersed along the rhizomycelium. Many of these cells appeared empty, while others contained light yellow globules. Some cells developed into zoosporangia, in which one half of the two-celled structure enlarged significantly in proportion to the other half, and became spherical, 15–20 µm in diameter. As they matured, sporangia assumed a granular appearance, and were light gold in color. Zoospore discharge was not observed. Resting spores were thick-walled, spherical or angular, 10–15 µm in diameter, and golden in colour.

**Karlingiomyces dubius** (Karling) Sparrow (1960). Fig. 16. [ch, 1]. Observed in two soil samples from a single site, this chytrid exhibited characteristics that conformed to the type. Sporangia were spherical or subspherical, 15–70 µm in diameter. Most sporangia had a single broad exit papilla, with a substantial hyaline region below the papilla. Rhizoids were generally isodiametric, and were extensive and profusely branched. Zoospore discharge followed a slow liberation of the operculum, which persisted on the sporangium following dehiscence. Zoospores remained quiescent for a few moments at the exit orifice prior to becoming motile.

**Lacustromyces sp.** Figs 17, 18. [ch, 4, 11, 13]. This polycentric chytrid was observed on chitin bait in soil samples from three collection sites. It lacked the spindle organs of other certain polycentric genera (e.g., *Novakoweskiella* and *Cladochytrium*), and was similar in gross thallus morphology to *Lacustromyces hiemalis* (Longcore 1993). The robust, extensively branched isodiametric rhizomycelium bore intercalary zoosporangia that developed multiple discharge tubes. Neither zoospore discharge nor resting spores were observed. Until additional observations and analysis clarify the taxonomic position of this organism, it is placed provisionally in this genus.

**Phlyctochytrium aureliae** Ajello (1945). Fig. 20. New record. [p, 3, 4]. This fungus was not abundant when it occurred in soil samples. Sporangia occasionally were spherical (18–25 µm in diameter) or subspherical, but generally were elongate (12–20 µm broad × 16–28 µm tall), with discernable dentate sporangial ornamentation. Zoospores were discharged singly or in small clusters. In older cultures, empty sporangia were common, but resting spores were not observed.

**Phlyctochytrium circulidentatum** Koch (1969) in Umphlett & Koch (1969). Fig. 21. New record. [p, 4]. This fungus was found sparingly in one soil sample from a single collection site. The subspherical to subelliptical sporangia were ornamented with a single lateral whorl of teeth. The sporangial size (18–26 µm in diameter), the lateral location of the prominent dentate sporangial ornamentation, and the presence of an apophysis confirm the identification of this chytrid.

**Phlyctochytrium indicum** Karling (1964). Figs 22–24. New record. [c, p, 1, 2, 3, 4, 10]. This chytrid was widespread in soil samples, and abundant where present. It exhibited substantial morphological variation from Karling's original description, particularly with respect to the rhizoidal system. Although many sporangia were sessile (fig. 22), as per the type, it was not unusual in the Australian material to observe sporangia that were interbiotic, with an inflated tubular (fig. 23) or apophysate (fig. 24) rhizoidal axis, in addition to the endobiotic apophysis. Zoospores were discharged in a vesicle outside the sporangium, where they remained quiescent for a short period of time before dispersing. As well as infecting pollen, this fungus occurred to a lesser extent on cellulose.

**Phlyctochytrium mucronatum** Canter (1949). Fig. 25. New record. [p, 3, 4, 10, 11]. This chytrid occurred abundantly in several soil samples. The spherical sporangia (18–36 µm in diameter) were ornamented laterally with numerous, small dentate enations and a blunt apical spine. The rhizoidal system consisted of an interbiotic rhizoidal stalk and a spherical apophysis within the substrate.
generally were smaller than those described by Willoughby. The material here does not closely match any Rhizophlyctis sp., which also occurred on chitin; however, the sporangia of material here
between the discharge tubes (1-4) and the spore mass. This organism closely resembles a chytrid described by

Isodiametric rhizoids originated from 3-20 broad axes spaced about the sporangium, and extended to more than
was easily observed on cellulose substrate, and in pure culture its colour was a deeper rose than that of material
elongate (20 um broad x 30 um tall), pyriform or irregular, and were numerous and abundant on chitin substrate.
sporangia were golden to reddish brown with verrucose walls. However, sporangia assumed exceptional large

distinctive hyphochytrid was abundant whenever found, and usually occurred on all substrates when present. The

Phlyctochytrium reinboldtiae Persiel (1959). Fig. 26. [p, 2, 4]. This isolate corresponded well with the type
material. The sporangia were subglobose to broadly ellipsoidal, with 3–12 cone-shaped discharge tubes that
often were tipped with a small hyaline globule prior to zoospore discharge. Occasional sporangia were interbiotic
with a stout extramatrical rhizoidal stalk. Zoospores were discharged into an exogenous vesicle. A large,
spherical, endobiotic apophysis that often filled the body of the pollen grain was observable in both pine and
sweet gum pollen.

Phlyctochytrium sp. Fig. 27. [p, 3, 10]. The elongate sporangia (14–24 μm broad × 16–30 μm tall) of this
organism were ornamented with distinctive and elongate (up to 8 μm in length) bicomute teeth. Sporangia were
subtended by an interbiotic rhizoidal stalk, and a spherical apophysis was visible within the pollen substrate. This
chytrid is possibly an additional morphotype in the species complex with Phlyctochytrium aureliae, P. mucronatum, and 'Miller's Dentate'.

Powellomyces sp. Figs 28, 29. [p, 4]. This soil-bom organism closely resembled Powellomyces (Longcore 1995). The endobiotic chytrid occurred in pollen grains, and zoospores were released via 1 or 2 discharge tubes. Occasionally zoosporangia developed exogenously from encysted zoospores. Sporangia were spherical (20–30 μm in diameter) to irregular (15–20 μm broad × 25–30 μm tall) in shape with single or multiple rhizoidal axes arising from 1 or 2 (–5) points on the sporangium. Neither zoospore discharge nor resting spores were observed. The organism is placed provisionally in this genus pending further observations.

Rhizidium richmondense Willoughby (1956). Fig. 30. New record. [c, 7]. This chytrid was abundant on cellulose substrate, and corresponded well with the type material. Sporangia were spherical, subspherical, or occasionally slightly irregular in profile. A zone of clear cytoplasm occurred between the prominent apiculus and the mass of zoospores within the sporangium. The rhizoidal system consisted of thin, sparsely branched rhizoids that originated from a small swelling at the base of the sporangium and extended several hundred microns into the substrate. Zoospores were discharged passively as a mass, and they remained quiescent at the exit orifice for a few moments prior to dispersal.

Rhizidiomyces bullatus (Sparrow) Karling (1944). Fig. 31. New record. [c, ch, k, p, 2, 3, 4, 10, 11]. This distinctive hypochytrid was abundant whenever found, and usually occurred on all substrates when present. The sporangia were golden to reddish brown with verrucose walls. However, sporangia assumed exceptional large size (60–100 μm in diameter) on chitin and keratin, as opposed to a much smaller average dimension (12–32 μm in diameter) on pollen and cellulose. Zoospore discharge was not observed.

Rhizophlyctis rosea (deBary & Woronin) Fischer (1892). Fig. 32. [c, 2, 4, 5, 6, 7]. This large, distinctive chytrid with sporangia up to 250 μm in diameter and occasionally visible without microscopy, is perhaps the most ubiquitous member of Chytridiomycota in soils worldwide (Sparrow 1960, Willoughby 2001). The fungus was easily observed on cellulose substrate, and in pure culture its colour was a deeper rose than that of material isolated by the authors in the United States (unpublished data).

Rhizophlyctis sp. Figs 35, 36. [ch, 1, 2, 3, 6, 7, 13]. Sporangia were spherical (20–52 μm), subspherical, elongate (20 μm broad × 30 μm tall), pyriform or irregular, and were numerous and abundant on chitin substrate. Isodiametric rhizoids originated from 3–20 broad axes spaced about the sporangium, and extended to more than 100 μm through the substrate. Prior to zoospore discharge, a plug of hyaline material occupied the regions between the discharge tubes (1–4) and the spore mass. This organism closely resembles a chytrid described by Willoughby (1965) as Rhizophlyctis sp., which also occurred on chitin; however, the sporangia of material here generally were smaller than those described by Willoughby. The material here does not closely match any
previous description, but as morphological limits of species in *Rhizophlyctis* commonly are uncertain, it is placed in the genus without erecting a new species to accommodate it.

*Rhizophlyctis coronum* Hanson (1944). Fig. 37. [p, 1, 2]. The gelatinous corona that surrounds the sporangium is unique among the members of *Rhizophlyctis*. This fungus occurred in only two samples, one from wet sclerophyll soil and one from subtropical rainforest soil. Its occurrence in this study agrees with Willoughby (1965), in which this chytrid was associated with impoverished soils exhibiting high microfungal diversity. For both the type material (Hanson 1944, 1945) and Willoughby's collection, the fungus occurred on cellulose; in this study it occurred on pollen, but not the cellulose substrate.

*Rhizophlyctis elyensis* Sparrow (1957). Fig. 38. [k, 4]. This fungus was encountered from a single soil sample. The angular sporangium, passive discharge of zoospores as a mass, and occurrence on keratin substrate conform to the type description.

*Rhizophlyctis globosum* (Braun) Rabenhorst (1868). Fig. 39. [p, 1, 2, 4, 5, 8, 13]. This chytrid was common and was observed in several soil samples from the many collection sites where it was found. Sporangia were 22–42 μm in diameter, the sporangial wall was double-contoured, and 2–4 exit papillae protruded on the upper half of the sporangium. As characteristic spiny resting spores were not observed, placement here is tentative.

*Rhizophlyctis macroporosum* Karling (1967). Fig. 40. New record. [ch, p, 1, 2, 3, 4, 11, 12]. This fungus was identifiable by the large sporangia (30–60 μm diameter), the conspicuous broadly conical exit papillae, and the simultaneous discharge of masses of zoospores through several exit papillae.

*Rhizophlyctis macrosporum* Karling (1938). Fig. 41. [p, 1, 2, 10]. This large *Rhizophlyctis* occurred sparsely, often being interbiotic with rhizoidal branches entering several pollen grains. Sporangia were 60–90 μm in diameter, and low, inconspicuous discharge papillae were observed only occasionally. Initial zoospore discharge occurred simultaneously from one or more discharge pores. Zoospores remained quiescent in an exogenous vesicle for 1–2 minutes before dispersing; the remaining zoospores then swarmed within the sporangium and rapidly dispersed one by one.

*Rhizophlyctis obpyriformis* (Karling) Karling (1977). Fig. 42. New record. [1, 2, 4, 7, 14]. This parasite was common, occurring predominantly on *Rhizophlyctis subangulosum* and to a lesser extent on *R. pollinis-pini*, and in both hosts the sporangial contents rapidly disintegrated following infection.

*Rhizophlyctis pollinis-pini* (Braun) Zopf (1887). Fig. 43. New record. [p, 1, 2, 3, 4, 5, 7, 8, 10, 11, 12]. This chytrid conformed to the type description. Sporangia were spherical, 10–25 μm in diameter, with a single broad discharge papilla. The rhizoids were extensive and branched. Zoospores were observed to discharge slowly and singly, and the empty sporangium was urn-shaped with wall material slightly recurved from the broad discharge pore.

*Rhizophlyctis sphaerotheca* Zopf (1887). Fig. 44. [p, 1, 2, 3, 4, 5, 7, 9, 10, 11, 12]. This fungus was common on pollen bait, with spherical sporangia generally larger (17–35 μm in diameter) than those of *R. pollinis-pini*. Zoospores were discharged singularly or in groups.

*Rhizophlyctis stipitatum* Sparrow (1957). Fig. 45. New record. [p, 1, 4, 8, 11, 12, 13]. The fungus was distributed widely among collection sites and habitats, yet was not abundant when encountered. The majority of sporangia exhibited a single discharge pore, and following zoospore discharge into an exogenous vesicle, the sporangial wall partially collapsed.

*Rhizophlyctis subangulosum* (Braun) Rabenhorst (1868). Fig. 46. [c, p, 1, 2, 4, 5, 10, 13, 14]. This chytrid was perhaps the most abundant *Rhizophlyctis* observed, and occurred in all habitats studied. The sporangia tended to be relatively large (25–40 μm along the greatest axis), and zoospore discharge was characteristic of the taxon. A mass of zoospores initially burst through a single exit papilla, remained quiescent for 20–90 seconds, and then began to disperse. Subsequently, the remaining zoospores within the sporangium began to swarm, and then exited singly from one or several discharge pores, and the sporangium was vacated quickly. Occurrence on pollen was most common, with somewhat smaller sporangia occurring on cellulose substrate.
**Septosperma rhizophydii** Whiffen (1942). Fig. 47. [1, 3, 4, 10]. This parasite was encountered infrequently, although it was distributed widely among various habitats. Willoughby (1965) mentioned the organism only once, as occurring in peaty soil in association with Scented Paperbark (*Melaleuca squarrosa*). In our material, the resting sporangia exhibited two distinct morphologies. At the Morton NP, Mount Bushwalker site, the resting sporangia were short and less than 14 μm in length, while at the remaining sites where it was found, resting sporangia concurred with the type material, being 16–25 μm in length. The chytrid was parasitic on *Rhizophydium pollinis-pini* and *R. sphaerotheca*.

**Spizellomyces kniepii** Gaertner ex D. Barr (1984). Fig. 48. [p, 1, 4]. Sporangia were 20–25 μm in diameter, with 12–50 narrow discharge tubes, and a spherical endobiotic apophysis. This chytrid occurred sparsely in several samples from two collection sites.

**Species diversity**

The greatest number of species occurred at collection site # 4, the dry sclerophyll forest of Ku-ring-gai Chase NP, the Duckholes, where 24 of the 38 identified taxa were found (Table 2). In both the subtropical rainforest (site # 1, Ourimbah, Palm Grove) and wet sclerophyll forest (site # 2, Windsor, Valley Centre), 18 of the identified taxa occurred, while 14 taxa occurred in open heath (site # 3, Ku-ring-gai Chase NP, Basin Track). Those four collection sites, each representing one of the four habitats investigated, were also the sites from which a significant number of samples were obtained. Among the other 10 collection sites, where sample size varied from 2 to 9 samples per site, between 1 and 12 species were observed per site.

Five species (*Chytromyces hyalinus, Phlyctochytrium indicum, Rhizophydidium macroporosum, R. pollinis-pini* and *R. sphaerotheca*) were found in all four collection sites where significant sampling occurred. Seven other species (*Chytromyces annulatus, Rhizidiomyces bullatus, Rhizophlyctis* sp., *Rhizophydium globosum, R. obpyriformis, R. subangulosum* and *Septosperma rhizophydii*) were found in three out of four of the collection sites where significant sampling occurred.

**Table 2. Species diversity in all collection sites.**

<table>
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<th>Collection sites</th>
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The focus of this study was to assess the biodiversity of chytrids in soils from four distinct vegetation types in central eastern New South Wales, using an established protocol. The vegetation types were dry sclerophyll forest, wet sclerophyll forest, subtropical rainforest and open heath. Soil samples were recovered from a total of 14 collection sites. A significant number of soil samples (n = 40–48) were obtained from each of four specific collection sites, and the equivalent sample size per site conveyed sampling parity to those four sites. The same four collection sites also individually represented one of the four vegetation types studied for the assessment of chytrid diversity. Conversely, from the remaining 10 collection sites, the low number of samples (n = 2–9 samples per site) precluded the inclusion of those sites in the assessment of chytrid diversity among the four vegetation types studied.

The evaluation of species diversity indicated that, among the four vegetation types investigated, the greatest number of chytrid species (25 of 38 identified taxa) was found in dry sclerophyll forests. Dry sclerophyll forests compose the largest proportion of eastern New South Wales bush, and make up more than half of Australia’s native forest, occurring in every State and Territory (Parish 2001). That chytrid biodiversity would be greatest in the most expansive vegetation type of the State is a reasonable expectation, assuming that it provides opportunities for diverse or varying growth conditions. At the other extreme, the lowest number of chytrid species (13 of 38 identified taxa) was found in open heath, the vegetation type that is the scarcest of the four habitats in New South Wales. Australian heaths are among the most species-rich plant communities in the world.

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Two sporangia with multiple rhizoidal axes originating from several points on the sporangium. Cluster of maturing sporangia on chitin. Figs 35, 36. Fig. 36. A long discharge tube. Rhizophlyctis sp. (Page 111).

Eight of the taxa isolated in this study represent newly observed organisms (Allomyces sp., Blyttiomycetes sp., Chytriomyces clade #1, Chytriomyces sp., Cladochytrium sp., Lacostromyces sp., Powellomyces sp. and Rhizophlyctis sp.), and they provisionally have been assigned generic affiliation based on currently available data. More complete observations and data on development, morphology, zoospore ultrastructure and molecular character are necessary before these entities may be delineated more accurately. In addition to the eight newly observed taxa, 17 other species represent new records for chytrids in Australia.

Thirteen of the species identified here also were found in one or more previous investigations (Harder & Gallwitz-Uebelmesser 1959, Karling 1988, Willoughby 1965). In total, 111 species of Chytridiomycota have been isolated from Australian soils. Only one species, Rhizophlyctis rosea, occurred in all four studies, attesting to the ubiquity of that organism.

Many of the taxa observed in this study, as well as the taxa recorded in the previous inventories of chytrids in Australian soils, exhibit a cosmopolitan distribution that is indicative of ancient origins of these organisms. Chytridiaceous fungi have a fossil record from approximately 400 Myr (4 x 10^6 years, Taylor et al. 1992), and these organisms may have existed as much as 1 Byr (10^9 years) ago (Simon et al. 1993). Thus, chytrids may have dispersed across Pangaea by the time of the separation of Pangaea into the supercontinents Laurasia and Gondwana approximately 80 Myr in the past. Subsequently, a well established chytrid flora should have existed in Meganesia (the then contiguous land masses of Australia, New Guinea and Tasmania) at the time of the Meganesian continental separation from Antarctica approximately 40 Myr in the past.

Given the long history of continental isolation, the potential for species evolution in Australia is great. With the exception of the newly described taxa in this study, none of the microfungal entities observed here appear to be endemic to Australia. However, in this study, the morphological variations expressed by Chytriomyces annulatus, P. indicum and Phlyctochytrium sp., as well as the general rarity of the newly described taxa, best exemplify the potential for species divergence. Additionally, morphological similarity of fungi with cosmopolitan distribution may mask greater variation at the cellular and molecular level. A concerted effort of comparison of species in pure culture, considering environmental, morphological, ultrastructural and molecular enquiries, may help to resolve this significant question.

The principal implication of both the biodiversity evaluation of this study, and the inventory analysis presented here of four surveys accomplished over the past 40 years, is of a vast and unrealized biodiversity of chytrids in Australian soils. Most of the earlier studies consisted of a limited and unstructured sampling regime. Harder & Gallwitz-Uebelmesser (1959), for example, examined 34 samples from Western Australia, South Australia, New South Wales, Queensland and Tasmania. Willoughby (1965) examined an undisclosed number of samples (perhaps no more than 50) from 12 or more sites, predominantly in Victoria. Karling (1988) examined 49 soil samples.
samples from 13 sites across the continent. The present study examined 227 soil samples from 14 sites in eastern New South Wales, the majority of which were within a 70 km radius of Sydney. That only one chytrid—Rhizophlyctis rosea—was common to the four inventory studies, while the remaining 110 identified taxa were scattered among 350 soil samples from 50 collection sites sampled at random across the continent, suggests that chytrid diversity in Australia is practically unknown and unexplored.

Chytrids occur in virtually all habitats on earth (Powell 1993). Australian natural habitats such as temperate rainforest, tropical rainforest, sedgeland swamps, grassland savannas, alpine moors, and freshwater environments have been sampled only sparsely, if at all, in any of the previously mentioned studies. No structured surveys of biodiversity in specific forest types such as those dominated by Callitris, Acacia, Casuarina, Melaleuca or Avicennia have been undertaken. Nor have soils been sampled from agricultural regimes, sites of volcanic activity or areas affected by fire or deforestation, and nothing is known about the effect of these disturbances on chytrid biodiversity. Additional studies are needed that can address the biodiversity of these organisms within these environments, habitats, and perturbations.

Acknowledgements
The authors gratefully acknowledge Dr Frank H. Gleason, Sydney, for invaluable assistance in locating collecting sites; and Mr David Tribe, Sydney and Mrs Pam O'Sullivan, Ourimbah, for giving their time and assistance in collecting samples. This study was supported by NSF-PEET Grant # DEB-9978094, and the Aquatic Ecology and Systematics Graduate Enhancement Program, The University of Alabama.

References


CHARACTERISATION OF FLAVOUR AND TASTE COMPOUNDS IN AGARICUS BLAZEI MURRILL SENSU HEINEM., THE CULTIVATED ALMOND MUSHROOM

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Maria Angela L. de A. Amazonas, Centro Nacional de Pesquisa de Florestas, Embrapa Florestas, Colombó, Parana, Brazil.
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Abstract

Agaricus blazei Murrill sensu Heinem. is a new cultivated medicinal and gourmet mushroom which is currently popular in Brazil, Japan and China. It is also cultivated in the USA, and it has recently drawn the attention of European mushroom growers. Upon investigating the mushroom’s pleasant almond flavour, it was observed that benzaldehyde and its precursor benzoic acid were the major components of the volatile fraction. Other benzylic compounds contributing to the flavour were benzyl alcohol, methyl benzoate and 4-hydroxybenzaldehyde. When reconstituting the commercially available dried mushrooms, almond flavour develops, presumably by enzymic conversion of benzoic acid to benzaldehyde and benzyl alcohol. Since benzoic acid is present at concentrations of 1280–3100 mg/kg dry weight, it may contribute to the excellent shelf life of the mushroom. Interestingly, benzoic acid also occurs in several close relatives of A. blazei, suggesting that this compound could well be a taxonomic marker. Among the non-volatile taste compounds, mannitol predominated to the extent of 22% on dry weight. Contents of taste-enhancing free glutamic and aspartic acids were comparable to those reported in the White button mushroom (Agaricus bisporus). The mycelium of A. blazei was found to be poor in nearly all compounds investigated. No almond flavour was observed and its crude protein content was only 13% compared with an average value of 30% in the dried mushrooms. Moreover, it had less than 1% of mannitol and only very low levels of free amino acids. Typical secondary metabolites as urea, free tryptophan and agaritine were even totally absent.


Introduction

Agaricus blazei Murrill sensu Heinem. (Heinemann 1993) is a new cultivated edible mushroom that is already popular in Brazil, Japan and China. It has recently turned up in the USA, where Paul Stamets (2000), the well-known American mushroom grower, has rapidly mastered its culture. The mushroom is an excellent edible, having a pleasant almond taste and a texture that is much better than that of any other agaric, including the White button mushroom, Agaricus bisporus, also known as the ‘Champignon de Paris’. Agaricus blazei Murrill has a variety of common names. In Brazil it is called Cogumelo do Sol (Mushroom of the sun), whereas the Japanese refer to it as Himematsutake. Stamets, capitalising on the popularity of the robust Portobello button mushroom in his country, has proposed the name ‘Almond Portobello’.

At present, the mushroom is most widely used for its medicinal virtues. It is said to contain 4 to 6% beta glucans, immuno-potentiating polysaccharides that also inhibit the growth of malignant tumors. Indeed, dried A. blazei and its medicinal preparations are widely advertised on the Internet. Still, the mushroom has definitely a future as a gourmet mushroom, because of its excellent nutritional qualities and large gastronomic potential. Commercial cultivation has been established in several countries, including China and Korea, which means that the price will probably soon come down. In a number of European research centres cultivation of A. blazei has been initiated with encouraging results. A general article about this mushroom and its domestication was recently published by Stijve & de A. Amazonas (2002).

In this paper the results of an investigation of both the volatile flavour compounds and the non-volatile taste components of A. blazei is reported. Although Chang et al. (2001) analysed its mycelia for monosodium
glutamate and 5'-nucleotides, we have not found any reports on the composition of its volatile flavour in the available literature.

The mushroom, whether wild-growing or cultivated has a most agreeable almond odour, especially when freshly picked, but it is also noticeable in the dried mushrooms of commerce, when these are soaked in water prior to culinary preparation. Several members of the genus *Agaricus* (e.g. *A. augustus*, *A. subrufescens* and the related secotioid species *Gyrophragmium dunalii*) also possess a more or less pronounced almond aroma. This feature is associated with the presence of benzaldehyde, which has indeed been identified as a major volatile compound in the said mushrooms (Chen & Wu 1984, Rapior et al. 2000, Wood et al. 1990). Even the ordinary cultivated white mushroom, *A. bisporus* contains benzaldehyde (Cronin & Ward 1971), but in this species the strong 'mushroomy' odour of 1-octen-3-ol predominates (Cronin & Ward 1971, Dijkstra & Wiken 1976, Hanssen 1982, Tressl et al. 1982).

**Materials and Methods**

*Mycelia and mushrooms*
Dried mycelium and its corresponding mushrooms were obtained from Paul Stamets, Olympia, USA. Several dried cultivars were supplied by commercial growers in the Brazilian States of Parana and Minas Gerais. A collection of wild-growing *A. blazei* came also from Parana, made in a heap of mowed and decomposed grass at the National Forest Research Centre, Embrapa Florestas. Other agarics analysed were obtained in Switzerland and in France.

*Isolation and gas chromatography analysis of the volatile fraction*
Isolation of the flavour compounds was performed by simultaneous extraction and distillation, using pentane-diethyl ether 1:1 v/v as a solvent (Chen & Wu 1984, Romer & Renner 1974) Ten gram test portions were rehydrated with 100 ml water prior to extraction. Gas chromatography and mass spectrometric identification (GC-MS) were carried out under conditions similar to those as described by Rapior et al. (2000).

*HPLC determination of benzoic acid*
An aqueous extract of the test portions was clarified, diluted with methanol, and directly subjected to HPLC using a C-18 bonded silica gel column, and a phosphate buffer-methanol mixture as a mobile phase. Detection was by spectrophotometry at 227 nm (Stijve & Hischenhuber 1984).

*Free amino acids*
Free amino acids were extracted from the dried mycelium and mushrooms by refluxing several hours with 80% ethanol. After evaporation of the solvent at 40°C under reduced pressure, the remainder was analysed by classic ion exchange chromatography as described in the AOAC manual (Cunniff 1996).

*Soluble sugars and sugar alcohols*
Simple sugars and sugar alcohols were determined according to the manufacturer’s manual of the DX 500 DIONEX system equipped with an ED 40 electrochemical detector. Sugars and sugar alcohols were extracted with water, and separated by ion chromatography on an anion exchange column (CarboPack MA 1). Electrochemical detection of the eluted compounds was by means of a pulsed amperometric detector and quantification by comparison with the peak areas of a series of standards. In addition, a rapid semi-quantitative analysis of the polyols was performed by thin-layer chromatography (Andary et al. 1979).

**Results and Discussion**

*Volatile flavour compounds*
The volatile compounds identified in *A. blazei* can be divided in several groups (Table 1). Benzylid derivatives (benzoic acid, benzaldehyde, benzyl alcohol, methyl benzoate and 4-hydroxybenzaldehyde) predominated, whereas phenylethyl compounds were less important. The C-8 aliphatic volatiles (e.g. 1-octen-3-ol and derivatives) which are well-known as flavour constituents of many other mushrooms (Buchbauer et al. 1993), amounted only to 3% of the total surface of the GC signals. Several unidentified compounds were observed, but they seemed quantitatively less important.
Table 1. Volatile compounds (mg/kg dry weight) in *Agaricus blazei*.

<table>
<thead>
<tr>
<th></th>
<th>Cultivar from Paraná, Brazil, Sample 01</th>
<th>Ditto from Minas Gerais, Brazil Sample 07</th>
<th>Wild-growing, collected in Embrapa Florestas, Colombo, Paraná, Brazil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity of Almond flavour</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Major volatiles*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>2430</td>
<td>428</td>
<td>885</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>2890</td>
<td>2250</td>
<td>1560</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>375</td>
<td>254</td>
<td>180</td>
</tr>
<tr>
<td>Methyl benzoate</td>
<td>220</td>
<td>107</td>
<td>58</td>
</tr>
<tr>
<td>4-hydroxy-benzaldehyde</td>
<td>116</td>
<td>86</td>
<td>99</td>
</tr>
<tr>
<td>Other constituents**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-phenyl ethanal</td>
<td>110</td>
<td>65</td>
<td>48</td>
</tr>
<tr>
<td>2-phenyl ethanol</td>
<td>75</td>
<td>72</td>
<td>65</td>
</tr>
<tr>
<td>2-phenyl acetic acid</td>
<td>90</td>
<td>110</td>
<td>42</td>
</tr>
<tr>
<td>C-8 compounds***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-octen-3-ol</td>
<td>42</td>
<td>38</td>
<td>45</td>
</tr>
<tr>
<td>1-octen-3-one</td>
<td>45</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td>3-octanol</td>
<td>20</td>
<td>20</td>
<td>n.d.</td>
</tr>
<tr>
<td>3-octanone</td>
<td>18</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Aliphatic C5 + C6 alcohols****</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As presumed, benzaldehyde is undoubtedly responsible for the almond flavour of the mushroom. Cultivar 01 having the most pronounced odour contained almost six times more of this compound than cultivar 07 in which the flavour was weak indeed. Interestingly, wild-growing *A. blazei* had an intermediate almond odour and benzaldehyde concentration. It must be taken into account, however, that production of flavour compounds by mushrooms depends on the composition of the growth medium/substrate, growth conditions, different stages of growth, and genetic variations of the strains (Jong & Birmingham 1993). We paid special attention to the possible presence of two other compounds possessing a similar odour. However, GC-MS indicated absence of p-tolualdehyde, which gives the fungus *Mycoacia uda* its strong smell of bitter almonds (Sastry et al. 1980). In a separate test (Stijve & de Meijer 1999), the collections also proved negative for hydrocyanic acid, which is not only contained in several food plants, but also in a number of cyanogenic mushrooms. Benzyl alcohol and methyl benzoate probably contribute a sweet flowering note. Since the three samples also contained several unidentified volatiles at varying concentrations, their contribution to the overall flavour—although unlikely—cannot be ruled out at this moment. In all three samples benzoic acid was the major volatile compound, but since it is odourless, it does not contribute directly to the mushroom’s flavour. Chen & Wu (1983) have rightly pointed out that this compound may well be the precursor of benzaldehyde. They postulated the existence of a reductase system converting benzoic acid into benzyl alcohol and benzaldehyde in both *Agaricus subrufescens* and *A. bisporus*. Evidence for such a system was obtained by blending fresh ordinary white mushrooms with benzoic acid, whereupon the formation of the almond smelling volatiles significantly increased. Oddly, the authors did not report any benzoic acid among the volatile fraction of *A. subrufescens* (Chen & Wu 1984), but this compound was probably not eluted from the Carbowax 20M column the authors used in the GC-MS determinative step.

The enzymes involved in the biosynthesis of benzaldehyde apparently survive for a long time in mushrooms which have been dried at a temperature below 40°C. A one-year-old herbarium collection of wild-growing *A. blazei*, rapidly developed a pleasant almond odour when moistened with water. So did most 12–18 month old samples of *A. blazei* that had been powdered for analysis, and kept in the freezer. This reconstitution experiment failed on a mycelium sample, but this material did not even smell of almonds upon receipt. Since Rapior et al. (2000) reported both benzaldehyde and benzoic acid in *Gyrophragmium dunalii*, we examined some adult specimens of this secotioid mushroom that had been kept at -10°C for two years. Upon thawing and concurrent disruption of the tissue cells, the enzymes were apparently reactivated, and a faint but distinct almond odour developed.

It should be pointed out that benzoic acid is also formed by oxidation of benzaldehyde. Since *A. blazei* is dried after harvest on gauze wire in a stream of warm air, it is highly probable that much benzaldehyde is lost in this
process, either by volatilisation or by conversion to benzoic acid. This loss of flavour is compensated by the enzymic reaction proceeding upon reconstitution of the mushrooms, provided that the enzymes were not destroyed upon drying.

**Benzoic acid**

Since literature reports on the occurrence of benzoic acid in mushrooms are scarce indeed, we decided to analyse all available *A. blazei* and some samples of related mushrooms for this particular compound. Since the GC-MS method is too laborious for this purpose, we used the more straightforward HPLC procedure proposed by Stijve & Hischenhuber (1984). For this purpose, a 2.5 g test portion of the powdered dried mushroom was reconstituted in 20 ml water prior to extraction. After clarification, HPLC determination did not pose any problems. All samples contained co-extractives, contributing peaks to the chromatogram, but no interference was observed when subjecting the benzoic acid signal to diode array analysis. The results of this investigation involving 24 samples are listed in table 2.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ORIGIN</th>
<th>BENZOIC ACID CONTENT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. blazei</em> mycelium</td>
<td>Olympia, WA, USA</td>
<td>42</td>
</tr>
<tr>
<td><em>A. blazei</em> mushrooms, cultivated on cow dung</td>
<td>ditto</td>
<td>1760</td>
</tr>
<tr>
<td>Ditto, cultivated on saw dust</td>
<td>ditto</td>
<td>1540</td>
</tr>
<tr>
<td><em>A. blazei</em> mushrooms N = 7</td>
<td>Produced in the Brazilian States Paraná and Minas Gerais</td>
<td>1280–3100</td>
</tr>
<tr>
<td><em>A. bisporus</em>, white, N = 4</td>
<td>Switzerland</td>
<td>58–150</td>
</tr>
<tr>
<td>Ditto, brown, N = 4</td>
<td>ditto</td>
<td>90–370</td>
</tr>
<tr>
<td><em>A. bitorquis</em>, wild-growing</td>
<td>ditto</td>
<td>87</td>
</tr>
<tr>
<td><em>A. xanthodermus</em>, ditto</td>
<td>ditto</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>A. silvaticus</em>, ditto</td>
<td>ditto</td>
<td>&lt;20</td>
</tr>
<tr>
<td><em>A. silvicola</em> wild-growing</td>
<td>ditto</td>
<td>1850</td>
</tr>
<tr>
<td><em>A. augustus</em>, ditto</td>
<td>ditto</td>
<td>2540</td>
</tr>
<tr>
<td><em>Gyrophragmium dunalii</em>, wild-growing</td>
<td>Ile d’Oleron, France</td>
<td>1230</td>
</tr>
</tbody>
</table>

Apparently, formation of benzoic acid mainly takes place in the mushrooms, since the mycelium hardly contains any. All *A. blazei* samples, whether cultivated on cow dung, saw dust or bagasse, contain comparable quantities of this metabolite. Cultivated White mushrooms (*A. bisporus*) have low levels of benzoic acid, and the brown variety contains about twice as much as the white. Wild-growing edible *A. bitorquis* has also very little, and the compound was absent from *A. silvaticus* and *A. xanthodermus*. Interestingly, high amounts of benzoic acid were only present in *A. blazei*, *A. silvicola* and *A. augustus*, species belonging to the subsection *Arvenses*, which suggests that the compound could well be a taxonomic marker. That the rare *Gyrophragmium dunalii* contains a comparable quantity of benzoic acid is not surprising. Indeed, this secotioid mushroom and the representatives of the said section *Arvenses* have many morphological and chemical characteristics in common (Guinberteau 1999, Rapior et al. 2000, Stijve et al. 2001).

Since the benzoic acid concentration of the said mushrooms is on the average 2000 mg/kg or 0.2%, it may well act as a preservative, especially in *A. blazei*, which has an excellent shelf life (Stamets 2000). Furthermore, *G. dunalii* can be kept in the refrigerator (at 5–7°C) for as long as two months without apparent degradation (Guinberteau 1999). In addition, during HPLC analysis, these mushrooms produced several more UV-absorbing peaks suggesting the presence of phenolic compounds—one was tentatively identified as p-hydroxy benzoic acid—which probably also have a marked bactericidal action.

It is somewhat puzzling that benzoic acid has not yet been recognised as a significant constituent of these edible mushrooms. In a study of carboxylic and fatty acids in *Agaricus* mushrooms, Abdullah et al. (1994) found only a minor concentration of benzoic acid in cultivated *A. bisporus*, but about 100 times more in an *A. silvicola* extract. Unfortunately, these authors made no attempt to quantify the compound.

**Soluble sugars and sugar alcohols**

The principal sugars and polyols occurring in edible mushrooms are trehalose, glucose, mannitol and arabinol (Laub & Woller 1984), which may well contribute to the taste. The results of a comparative HPLC analysis of
mycelium and mushrooms grown on two substrates are reported in Table 3. Both mycelia and mushrooms have about the same low concentrations of glucose and fructose, but biosynthesis of trehalose and the polyols apparently mainly takes place in the fruit bodies.

Table 3. Soluble sugars and polyols in mycelium and mushrooms of A. blazei (percentage on dry weight).

<table>
<thead>
<tr>
<th></th>
<th>Mycelium</th>
<th>Mushrooms grown on saw dust</th>
<th>Mushrooms grown on bagasse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.</td>
<td>2.</td>
<td>1.</td>
</tr>
<tr>
<td>Arabitol</td>
<td>0,01</td>
<td>0,01</td>
<td>0,13</td>
</tr>
<tr>
<td>Trehalose</td>
<td>0,01</td>
<td>0,01</td>
<td>0,83</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0,75</td>
<td>0,74</td>
<td>17,83</td>
</tr>
<tr>
<td>Glucose</td>
<td>0,62</td>
<td>0,61</td>
<td>0,66</td>
</tr>
<tr>
<td>Fructose</td>
<td>0,07</td>
<td>0,06</td>
<td>0,06</td>
</tr>
</tbody>
</table>

These results are different from those reported by Chang et al. (2001) who found in the mycelium 3,14% arabitol, 4,53% glucose and 2,39% trehalose. The values for the sugars are higher than those we measured both in the mycelium and in the mushrooms. The high level of arabitol and the absence of mannitol reported by the Chinese scientists can only be explained by assuming an analytical error. Indeed, arabitol is a polyol that is typical for some subsections among the Boletales (Andary et al. 1979), but it is only a minor constituent of dark-spored gilled fungi like Agaricus species, which invariably contain much mannitol. This also applies to cultivated A. bisporus that has an average content of 20% mannitol on dry matter (Laub & Woller 1984).

TLC screening of other A. blazei samples revealed that arabitol content was often below detection, whereas mannitol concentrations were in the range of 15–22%. The sum of the sugar concentrations fluctuated between 0,5–3% on dry weight.

Free amino acids
The total free amino acid content of the mushrooms was about 10 times higher as that of the mycelium (Table 4). The presence of seven essential amino acids was noted. The sum of glutamic acid, glutamine, aspartic acid and alanine amounted to more than 70% of the total. Low levels of ornithine and γ-amino butyric acid were also observed in the mushroom extracts, but no quantitation was attempted. Interestingly, the free amino acid pattern of A. blazei mushrooms resembles that of the cultivated A. bisporus as reported earlier (Eisenhut et al. 1995, Kurkela et al. 1980).

Table 4. Free amino acid content (percentage on dry weight) of Agaricus blazei cultivars.

<table>
<thead>
<tr>
<th></th>
<th>MYCELIUM (N = 2)</th>
<th>MUSHROOMS (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0,05–0,12</td>
<td>0,62–0,97</td>
</tr>
<tr>
<td>Threonine*</td>
<td>0,04–0,05</td>
<td>0,08–0,12</td>
</tr>
<tr>
<td>Serine</td>
<td>~ 0,1</td>
<td>0,10–0,18</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0,02–0,10</td>
<td>1,28–1,75</td>
</tr>
<tr>
<td>Glutamine</td>
<td>n.d.</td>
<td>0,65–0,90</td>
</tr>
<tr>
<td>Proline</td>
<td>n.d.</td>
<td>0,05–0,12</td>
</tr>
<tr>
<td>Glycine</td>
<td>n.d.</td>
<td>0,03–0,08</td>
</tr>
<tr>
<td>Alanine</td>
<td>0,10–0,12</td>
<td>0,48–0,75</td>
</tr>
<tr>
<td>Valine*</td>
<td>0,08–0,15</td>
<td>0,10–0,16</td>
</tr>
<tr>
<td>Isoleucine*</td>
<td>~ 0,2</td>
<td>0,05–0,08</td>
</tr>
<tr>
<td>Leucine*</td>
<td>0,04–0,05</td>
<td>0,08–0,11</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>n.d.</td>
<td>0,12–0,21</td>
</tr>
<tr>
<td>Histidine</td>
<td>0,02–0,03</td>
<td>0,05–0,07</td>
</tr>
<tr>
<td>Lysine*</td>
<td>0,04–0,06</td>
<td>0,11–0,13</td>
</tr>
<tr>
<td>Phenylalanine*</td>
<td>~ 0,01</td>
<td>0,10–0,14</td>
</tr>
<tr>
<td>Arginine</td>
<td>0,03–0,05</td>
<td>0,15–0,32</td>
</tr>
<tr>
<td>Tryptophan*</td>
<td>n.d.</td>
<td>0,05–0,12</td>
</tr>
<tr>
<td>TOTAL</td>
<td>0,45–0,77</td>
<td>4,10–6,21</td>
</tr>
</tbody>
</table>

n.d. = not detected, * essential amino acids.
As Yamaguchi (1979) has rightly pointed out, the free amino acids aspartic and glutamic acids are mainly responsible for bringing out the umami taste in mushrooms. These monosodium glutamate-like (MSG-like) amino acids are present at appreciable concentrations and contribute therefore to the palatable taste of A. blazei.

The results for most of the free amino acids in the mycelium agree rather well with those reported by Chang et al. (2001), although these authors could not detect any glutamic acid. Summarising it can be said that the mycelium’s crude protein content was only 13% compared with an average value of 30% measured in the dried mushrooms. Moreover, noting absence of free tryptophan in the mycelium and its relative high content in the mushrooms, we subjected a methanolic extract to TLC analysis for other typical secondary metabolites (Stijve et al. 1986). Not surprisingly, urea and agartinine, which amounted both to 0.5–1% in the mushrooms, were found to be conspicuously absent from the mycelium.

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References


ERRATUM
The Australasian Mycologist, Issue 22 (2)

This article was published in error as a report. It should have been published as a refereed research paper.

EIGHTH INTERNATIONAL MYCOLOGICAL CONGRESSES, CAIRNS, 20–26 AUGUST 2006

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