

CHAPTER THREE

3. CHARACTERIZATION OF TWO LOCALLY GROWN *GANODERMA* SPP.

3.1 Introduction

The fruiting bodies (basidiocarps) of *Ganoderma* spp. have similar morphological characteristics that have caused confusion in identification (Adaskaveg and Gilbertson, 1986). Numerous species have been described in temperate regions and many synonyms have been reported (Moncalvo *et al.*, 1995). Murrill (1902) was the first to consider the primary taxonomic characters for the *Ganoderma* spp. to be host specificity, geographical distribution, and morphology of the fruiting body. The latter included context color, the shape of the margin of the pileus, and whether the fruiting body was stipitate or sessile.

Subsequently, Nobles (1948, 1958, 1965) published a series of multiple-choice keys for the identification of wood-inhabiting Hymenomycetes (including *Ganoderma* spp.). The diagnostic characters were based on cultural characteristics which include presence or absence of extracellular oxidase, type of septation of hyphae, occurrence of special structures and accessory spores, colour of hyphae and mycelial mats, color changes in the agar, rates of growth, formation of fruit bodies, odor, host relationships, and interfertility phenomena. These cultural characteristics, coupled with recent microscopic characters of the basidiospores can provide an adequate identification of *Ganoderma* species (Adaskaveg and Gilbertson, 1986, 1988).

The basidiospores are sexual spores containing a complex double wall. The perisporium (outer wall) is connected to the inner wall by inter-wall pillars (Adaskaveg and Gilbertson, 1986, 1988). The spore is the most characteristic and distinguishing feature of the Ganodermataceae (Donk, 1964; Furtado, 1962). Basidiospores of species within *Ganoderma* are morphologically similar, but the spores have been shown to be varied in size within a species (Steyaert, 1977, 1980; Adaskaveg and Gilbertson, 1986). Specific differences in basidiospore morphology of several species have been reported using light microscope (LM) and scanning electron microscopy (SEM) (Pegler and Young, 1973; Adaskaveg and Gilbertson, 1986, 1988; Tham, 1998).

Isolates used in pharmaceutical and medicinal studies, and consequently commercially cultivated isolates are largely referred as *G. lucidum* (Moncalvo *et al.*, 1995). However, a closely related species, *G. tsugae* has been reported to be very similar in morphology to *G. lucidum* (Adaskaveg and Gilbertson, 1986). The objective of this experiment was to tentatively characterize two *Ganoderma* species obtained from a mushroom farm in Semenyih by a comparative study of the morphology of basidiospores and cultural characteristics. The result of this study will enable the farmers to better recognize their cultivated strains.

3.2 Materials and Methods

3.2.1 Fruiting body collections and descriptions

Two different *Ganoderma* species, sample A and B were collected in April 1999 from a mushroom farm in Semenyih, Malaysia. Both the mature fruit bodies were collected after about 2 months of cultivation. The colours of the fruit body, context and pileus were noted using the *Methuen Handbook of Colour* (1981).

3.2.2 Microscopic studies of the basidiospore

Basidiospore morphology was examined with light microscopy and scanning electron microscopy (SEM). Spore length, width, and index (length/ width) were determined for both species (Steyaert, 1980). For SEM observations, spores were obtained by washing the surface of sample A with distilled water onto a small Petri dish. For sample B, the spores were collected by cutting a piece of the inner context and tapping softly onto a small Petri dish containing small amounts of distilled water.

The spore suspensions were loaded onto a nuclear pore membrane (0.45 μm). The membrane was then fixed with 2% w/v aqueous osmium tetroxide (Sigma Chemicals) for 15 min. and washed thoroughly with distilled water. Washed materials were dehydrated through a graded series of alcohol (15 min. for each step) to absolute alcohol followed by two changes of absolute alcohol. The alcohol was replaced

gradually by acetone (30:70, 50:50, 70:30 parts of acetone: absolute alcohol) and the materials were then washed twice in absolute acetone.

Dehydrated materials were transferred, under acetone, to a critical point drying apparatus (C.P.D. Biorad, England) and critically point dried using liquid carbon dioxide. Dried materials were mounted on specimen stubs using double sided adhesive tape and coated with 500Å thick coating gold using a Cool E5100 diode sputter coater (Biorad, England). The materials were viewed in a Philips SEM 515 at a range of kV's.

3.2.3 Cultural characteristics studies

The cultural characteristics were studied according to Nobles (1965) and Adaskaveg and Gilbertson (1986).

Isolation of pure culture

Small pieces (4 mm x 4mm x 5 mm) of the context tissues of the basidiocarps were cut and washed several times with sterilized distilled water containing 5% of Chlorox before inoculating onto 1% yeast extract enriched Potato Dextrose Agar (PDA, Difco) plates. The plates were incubated at $27 \pm 1^\circ\text{C}$ until there was visible mycelial growth. The mycelium which grew out of the tissues was subcultured immediately onto fresh PDA plates.

Microscopic cultural studies

A small amount of mycelium was picked up with a straight needle from the growing edge of pure culture. The sample was then mounted on the glass slide using distilled water or lactophenol blue and cultural characteristics were determined under the light microscope.

Enzyme activity during growth

Discs of mycelium from pure cultures of sample A and B were inoculated in the center of Malt Extract Agar (MEA, Difco) plates containing tannic or gallic acid. The media were prepared by adding 0.5 % gallic acid or tannic acid to 1.5 % malt agar (Nobles, 1965). A brown zone around the colony indicates positive result for polyphenol oxidase activity.

Effect of temperature on growth rate

Discs (5 mm) of mycelium were cut 2 mm from the growing edges of the pure culture and inoculated onto MEA plate for temperature studies. Optimal growth temperature ranges were determined by growing isolates for one week on MEA at eight temperatures ranging from 17, 22, 25, 28, 30, 33, 37 to 40 °C. For each temperature tested, three replicate plates were setup.

3.3 Results and Discussion

3.3.1 Macromorphology of fruit body

Macroscopically, the two samples collected were clearly distinct in both form and colour. Sample A appeared bigger in size, laccate with fan-shaped basidiocarp and showed a gradient of yellow to reddish-brown colours. In contrast, the basidiocarp of sample B was smaller, non-laccate, with cloud-like basidiocarp and was light brown to brown colour (Plate 2).

The pileus and context colours were also noted to be different. The colour of the pileus was deep red in sample A, while in sample B, the pileus appeared brown (Plate 2). The context of sample A was white throughout and yellow brown at the edge of fruit body, while sample B was grayish-brown throughout and dark brown at the edge of fruit body (Plate 3). However, both samples were observed to be stipitate.

Another distinct feature was that the surface of basidiocarps of sample B was covered with spores, whereas sample A contained no spores on its surface. This may be due to the presence of bigger pore size observed on the context surface of sample B as compared to sample A (Plate 3). As a result, more spores will fall from the top of one fruit body to the surface of another fruit body (because of staking) during the cultivation of the mushroom (Plate 1h, i).

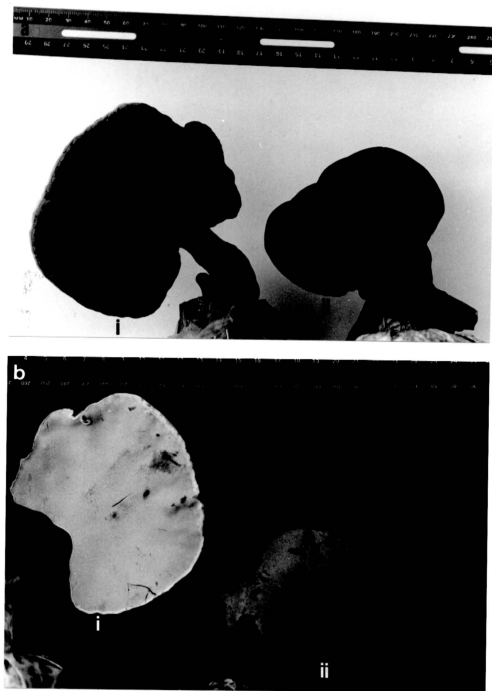


Plate 2: Two *Ganoderma* species collected from a mushroom farm in Semenyih. i. Sample A was claimed to be *G. tsugae*. ii. Sample B was claimed as *G. lucidum*. (a) Front view and (b) back view.

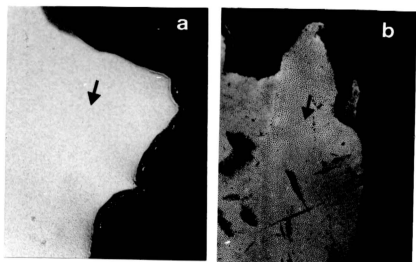


Plate 3: The context colour and pore size of the *Ganoderma* species. (a) Sample A appeared white and smooth with no visible pores and (b) sample B appeared grayish brown with visible round to angular pores.

3.3.2 Microscopic studies of the basidiospore

Basidiospores of sample A and B were brown and ovate, with a truncate to rounded apex, and an eccentric hilar appendix on a rounded base (Plate 4 and 5). The apex contained a germ canal from which germ tubes were observed to emerge (Plate 5). These features are characteristic of the *Ganoderma* basidiospores (Pegler and Young, 1973; Adaskaveg and Gilbertson 1986, 1988).

The spores from both species had a double wall with inter-wall pillars separating the two walls (Plate 4). Donk (1964) described this spore wall as consisting of an inner brown wall layer bearing spines that pierce an outer hyaline wall layer. Most of the spores were found to contain variable sizes of vacuole with high refractory index (Plate 4). Furtado (1962) described these structures as lipid drops and referred to them as guttulae.

Comparison of sample B basidiospores with those of sample A showed distinctive differences in their sizes and structures. Basidiospores of sample B were bigger compared to basidiospores of sample A (Table 3.1).

Table 3.1: Biometrics of basidiospores^a of two locally grown *Ganoderma* species.

Species	Mean length	Mean width	Range	Spore Index ^b
Sample A	10.10 ± 1.29	6.14 ± 0.80	9.13-13.28 x 5.81-8.30	1.64 ± 0.08
Sample B	15.06 ± 0.79	9.98 ± 0.92	14.00-16.25 x 9.00-11.25	1.50 ± 0.05

^a Measurements were determined from 20 basidiospores. (Appendix II)

^b Spore index (SI) = spore length/ spore width, $P = (0.01)$

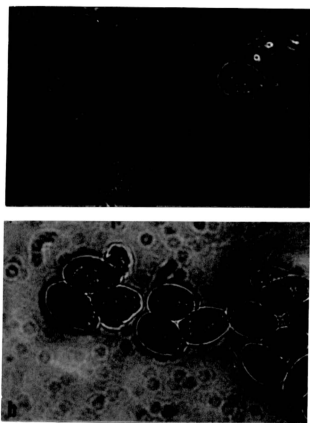


Plate 4: Light microscopy of basidiospores of the *Ganoderma* species. Basidiospores were brown and ovate. **(a)** Inter-wall pillars (arrow) of sample A are well defined. **(b)** Inter-wall pillars (arrow) of sample B are not as apparent as basidiospores of sample A



Plate 5: Scanning electron microscopy of the basidiospores. (a) The attachment of the spore to the sterigma via the germ tubes in sample A. (b) Mature spores with characteristic germ tubes observed in sample B.

The spore size of sample B ($15.06 \times 9.98 \mu\text{m}$) was also found to be larger than the range of reported range for *Ganoderma* spp., which was $8-13 \times 4.5-8 \mu\text{m}$ (Pegler and Young 1973). As noted by Pegler and Young (1973), exceptions do occur, particularly amongst the tropical species. For example, basidiospores of *G. ochrolaccatum* from Philippine Islands measured $27-32 \times 15-18 \mu\text{m}$, and basidiospores of *G. eminii* from East Africa, measured $26-35 \times 14-24 \mu\text{m}$.

The spore length and width were significantly different ($P = 0.01$) between the two samples (Table 3.1). Sample A with mean spore index (length/width ratio) of 1.64 was also significantly different ($P = 0.01$) from that of sample B, which was 1.50. The spore indexes calculated were similar to those described by Adaskaveg and Gilbertson (1986) who reported 1.50 for *G. lucidum* and 1.57 for *G. tsugae*.

No overlapping of sizes (Table 3.1) were observed between the two species, suggesting identification using a single character may be possible. However, Steyaert (1972) had demonstrated in *G. tornatum* that basidiospore size varies with latitude and altitude. He also noted that context colour became darker with southern latitudes and lower altitudes. These observations suggested the influence of temperature on morphological characters.

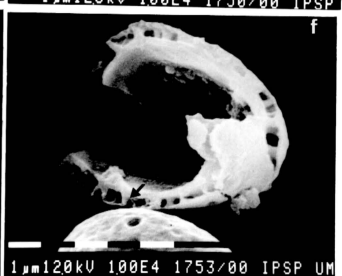
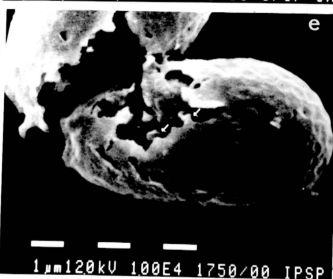
Furthermore, the spores of the sample A which were collected from the inner context of fruit body may include both the mature and immature spores as compared

to the mature spores of sample B, which were collected from the surface of the fruit body. Thus, using only the spore size may give rise to misidentification if no other criteria are taken into consideration.

Under light microscope, basidiospores of sample A had inter-wall pillars that were distinct partitions between the inner and outer walls, while the inter-wall pillars of basidiospores of sample B were not as obvious (Plate 4). In addition, the vacuoles were noted to be more conspicuous in spores of sample B as compared to spores of sample A (Plate 4).

Under the SEM, basidiospores of sample B were observed as 'smooth' walled, in spite of shallow, evenly spaced depressions (Plate 6d). The fractured basidiospores of sample B exposed narrow and numerous inter-wall pillars (Plate 6e, f). In contrast, the basidiospores of sample A were 'rough' walled, with pronounced depressions (Plate 6a). The inter-wall pillars of fractured spores were broader and less numerous than those of sample B (Plate 6b, c).

Based on the microscopic studies of the basidiospores, and comparing the results obtained with that of reported literatures (Pegler and Young, 1973; Steyaert, 1977; Adaskaveg and Gilbertson 1986, 1988), sample A and B were tentatively identified as *G. tsugae* and *G. lucidum* respectively.



3.3.3 Macroscopic cultural studies

The Petri plates (8.5 cm) were fully covered in one week and two weeks by the mycelium growth of *G. lucidum* and *G. tsugae* respectively. This result is in accordance to Nobles (1965) and Adaskaveg and Gilbertson (1986), except mycelium of *G. lucidum* was observed to grow faster in culture plates as compared to the two weeks mycelium growth reported for *G. lucidum* (Nobles, 1965).

Both the mycelial mats appeared light yellow to brown after 2 weeks of growth. Some cultural plates of *G. lucidum* were observed to produce primordia after five weeks of incubation at room temperature (Plate 7a, b and c). The primordia in culture appeared as white to light brown laccate projections from the white mycelium, with approximately 5 mm high and 3 mm wide. No production of primordia was noted in the cultures of *G. tsugae* even after 7 weeks of incubation (Plate 7a). Nobles (1965) and Adaskaveg and Gilbertson, (1986) have noted that some of their *G. lucidum* isolates produce primordia in the culture, whilst no production of primordia was found in *G. tsugae* isolates.

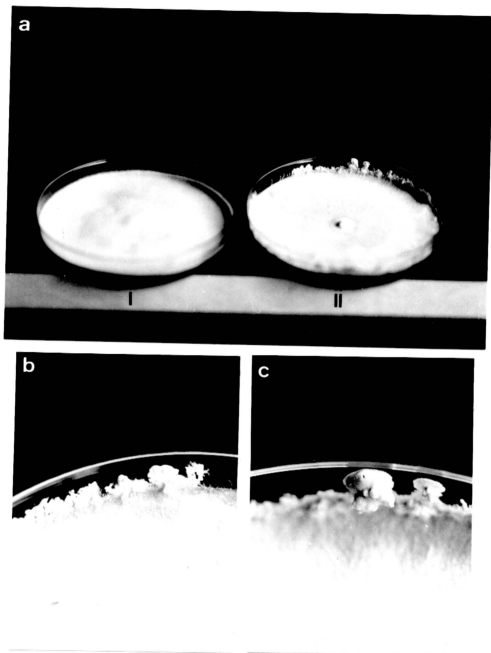


Plate 7: Appearance of the mycelium mat of *Ganoderma* spp. after five weeks of incubation at room temperature. (a) i. No production of primordia in *G. tsugae*, while ii. primordia was observed in *G. lucidum*. (b) and (c) Primordia of *G. lucidum* (close-up).

Host relationship cannot be determined due to lack of time. However, both samples, according to the farmer, grow equally well on softwood (rubber wood sawdust), with *G. lucidum* having shorter spawn running time (Kuan, 1999). The mycelial growth (spawn running) in the bags, according to the farmer, took around 2 ½ to 3 weeks, depending on the amount of rice bran being added to the substrate. The increase in rice bran addition to the substrate (i.e. a higher concentration of nitrogen) the faster the growth of mycelium and the earlier fruit body being formed (Kuan, 1999).

3.3.4 Microscopic cultural studies

Both the samples were observed to consist of thin-walled hyphae, with consistently nodose-septate (Plate 8a). The hyphae differentiated to form fiber hyphae and cuticular cells (Plate 8b). Clamp connections were also observed (Plate 8c) and these are characteristic of the basidiomycetous mycelia (Hudson, 1986).

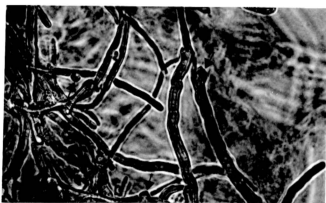


Plate 8: Microscopic characters of cultures of *Ganoderma* species observed under light microscope. (a) Thin-walled hyphae, with consistently nodose-septate (400x). (b) i. fiber hyphae and ii. cuticular cells (1000x).

Chlamydo spores were found to be present only in *G. lucidum* (Plate 9). The chlamydo spores are asexual spores primarily produced for survival rather than dispersal (Berry, 1988). In lactophenol blue, the chlamydo spores were bluish to hyaline and approximately 12-14.0 x 10.6-11.4 μm . This result correlates well with 12-20 x 10 -12 μm reported for *G. lucidum* by Adaskaveg and Gilbertson (1986). Nobles (1965) and Adaskaveg and Gilbertson (1986) have showed that all their *G. lucidum* isolates produced abundant chlamydo spores in culture, while none of the *G. tsugae* isolates produce chlamydo spores in culture. The wall of chlamydo spores was thick, with characteristic accumulation of vesicles which may be reserves of glycogen or oil (Hudson, 1986).

3.3.5 Enzyme activity during growth

Both *G. tsugae* and *G. lucidum* showed positive test results for polyphenol oxidase production (Plate 10). *G. lucidum* was found to produce a higher amount of polyphenol oxidase (3+), with diffusion zone light to dark brown, extending quite a distance beyond the margin of the mat. In contrast, *G. tsugae* only produced low amounts of polyphenol oxidase (1+), with light brown diffusion zone formed around the mat (Plate 10). The low level of polyphenol oxidase production by *G. tsugae* may be due to its inability to grow well under acidic condition of the medium. Further investigation in suitable medium is necessary to confirm the actual polyphenol activity of *G. tsugae*.

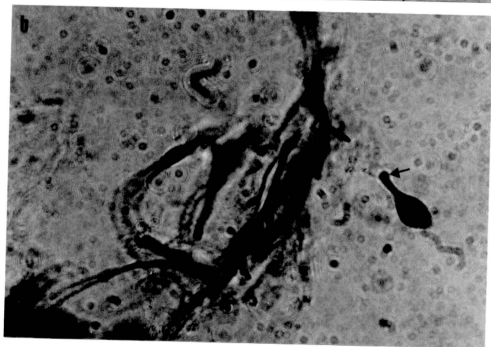
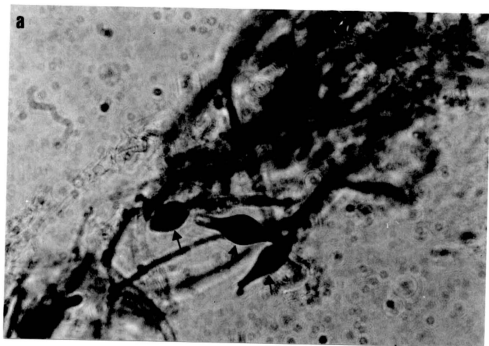


Plate 9: Chlamydospores observed only in *G. lucidum* under light microscope.
(a) Intact chlamydospores **(b)** Detached chlamydospore.

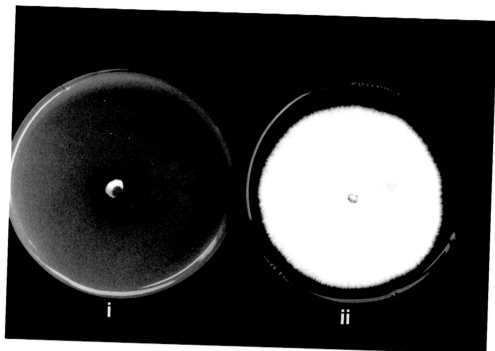


Plate 10: Production of extracellular enzymes by the *Ganoderma* species.

- i. Production of polyphenol oxidase by *G. tsugae*,
- ii. Production of polyphenol oxidase by *G. lucidum*.

3.3.6 Temperature studies

The temperature studies indicated different growth rates and optimal temperature ranges for the two species (Figure 3.1 and Plate 11). The rate of growth of *G. tsugae* and *G. lucidum* in culture plates increased differently with increasing temperature. *Ganoderma lucidum* had an optimal temperature range between 28-33°C, with average growth rate of 11.8 mm/day. In contrast, *G. tsugae* was observed to have a higher optimal temperature range of 33-37°C, with lower average growth rate of 6.8 mm/day (Figure 3.1).

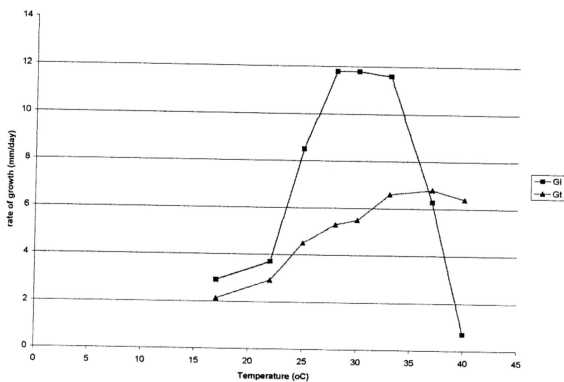


Figure 3.1: The growth rate of *G. tsugae* (Gt) and *G. lucidum* (Gt) at different temperatures.

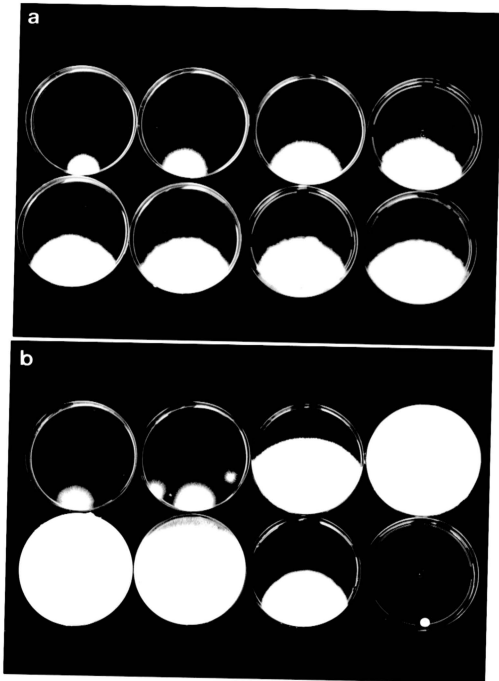


Plate 11: Linear growth of the *Ganoderma* spp. after 7 days at a range of temperature on MEA plates. **(a)** Linear growth of *G. tsugae* at the following temperatures (upper row, left to right) 17, 22, 25, 28, (bottom row, left to right) 30, 33, 37, 40°C. **(b)** Linear growth of *G. lucidum* at the following temperatures (upper row, left to right) 17, 22, 25, 28, (bottom row, left to right) 30, 33, 37, 40°C.

When temperature increased above the optimum temperatures, growth rate started to decline. A sharp decline of growth rate was found for *G. lucidum* above 33°C and at 40°C, no growth was observed. In contrast, growth of *G. tsugae* started to decline after 37°C (Figure 3.1).

Adaskaveg and Gilbertson (1986) have reported that *G. lucidum* had an optimal range between 30 and 34°C with growth rate from 7-11mm /day, which correlates well with our *G. lucidum*, which had an optimal temperature range between 28-33°C and average growth rate of 11.8 mm/day. However, our *G. tsugae* strain was capable of growing at 37°C when compared to the reported *G. tsugae* strain, which was not capable of growing above 34°C (Adaskaveg and Gilbertson 1986). Furthermore, the rate of growth of *G. tsugae* was also much faster (6.8 mm/day) as compared to 1-3 mm /day reported for *G. tsugae* (Adaskaveg and Gilbertson 1986).

One possible explanation for this is that the *G. tsugae* strain reported by Adaskaveg and Gilbertson (1986) was obtained from North America region, whereas the current strain was obtained from the subtropical region. Hence, the strain may have changed, adapting to growth at higher temperatures. These changes may include changes in amounts of lipids and in relative abundance of different classes of lipids in the mycelium. As a result, growth was observed to be higher than the reported values because the biochemical reactions will proceed at a faster rates at higher temperature (Griffin, 1994).

The lack of temperature effect on *G. tsugae* contrasted with a pronounced effect for *G. lucidum* over the same temperature range (33-40°C) (Figure 3.1) may also be due to the differences in amounts of lipids and/or in relative abundance of different classes of lipids in the mycelium. Further studies to understand the effect of temperature, especially temperature extremes, on the growth of fungi should be conducted. The availability of mutants in this regard is particularly important (Griffin, 1994).

The discussion of the results has so far been comparing the reported *G. tsugae* and *G. lucidum* with sample A and B. This is because many of the cultural characteristics and basidiospores morphology agree well with these two reported species. However, two other species, *G. valesiacum* and *G. resinaceum* from Europe were also very similar in morphology and temperature relationships with sample A and B as well as the North American cultures of *G. tsugae* and *G. lucidum*, respectively (Adaskaveg and Gilbertson 1986, 1988).

A review of the literature on the names of the species of *Ganoderma* indicated that *G. lucidum* and *G. resinaceum* were possible names for specimens obtained from hardwoods, having smooth basidiospores, and produced chlamydospores in culture. *Ganoderma valesiacum* and *G. tsugae* were possible names for specimens obtained from conifers, having rough basidiospores, and lacking chlamydospores in culture (Adaskaveg and Gilbertson, 1986)

The possibility of sample A and B being *G. valesiacum* and *G. resinaceum* cannot be ruled out. However, since both of these strains have so far being reported only from Europe, the chances may be low. Hence, on the basis of the available results (Table 3.2), sample A and sample B were tentatively identified as *G. tsugae* and *G. lucidum* respectively. In later experiments, sample A will be noted as *G. tsugae*.

Table 3.2: Summary results of comparative studies on sample A and B

Sample A (<i>G. tsugae</i>)	Sample B (<i>G. lucidum</i>)
Optimum temp. range of 33-37°C	Optimum temp. range of 28-33°C
Do not produce chlamydospores in culture	Produce chlamydospores in culture
Smaller basidiospores (6.14 x 10.10 µm)	Bigger basidiospores (9.98 x 15.06 µm)
'Rough' basidiospores	'Smooth' basidiospores
Inter-wall pillars are well defined	Inter-wall pillars are not well defined
Do not produce primordia in culture	Produce primordia in culture