Successful cultivation of a valuable wild strain of *Lepista sordida* from Thailand

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Abstract

Lepista sordida is an edible and medicinal mushroom, but until now it had to be collected from the wild. The present study is the first report of the successful cultivation of a wild strain of *L. sordida* from Thailand. The morphological description and molecular examination of the fungus are included, in order to confirm the identification of the species. Optimization was carried out for mycelium growth and fruiting body production. The optimal conditions for growing the mycelia on solid medium were yeast malt extract agar (YMA), pH 6.3 and a temperature of 25 °C. A sorghum mushroom spawn was used for upscaling of the mycelium to be used for fruiting body production. Optimal conditions for the fruiting phase were 25 °C with 95–97% humidity in a compost rice straw medium with sandy-soil casing layer. Additionally, the secondary metabolites of fruiting body and cultured mycelium were investigated. Nudic acid B, a known toxic polyacetylene, was isolated from submerged cultures of *L. sordida*, while no polyacetylenic compounds were found in the fruiting bodies.

Keywords: bioactive metabolites, cultivation, edible mushroom, molecular data, morphology

Introduction

Lepista is a clitocyboid agaric genus belonging to the Tricholomataceae. Approximately 50 species have been described in this genus, which is widely distributed over Asia, North America and Europe (Alvarado et al. 2015). However, recent molecular evidence (Moncalvo et al. 2002; Matheny et al. 2006; Alvarado et al. 2015) suggests that the genus *Lepista* is polyphyletic, with one core clade including the type of *Lepista*, *L. densifolia* (J. Favre) Singer & Clémençon, situated apart from a second clade containing other *Lepista* species, including *L. nuda* (Bull.) Cooke and *L. sordida* (Schumach.) Singer, as well as species of *Clitocybe*. It therefore appears that neither of the choice edible species, *L. nuda* or *L. sordida*, are phylogenetically closely related to the core group of *Lepista* (Alvarado et al. 2015; Vizzini et al. 2012). Rather they fall into one of several clades comprising a large heterogeneous and complex group of collybioid fungi called "Clade III *Collybia* and allies" which contains *Clitocybe, Collybia*, and *Lepista* spp. A close relationship between *Clitocybe* and *Lepista* already has been suggested and led some authors to consider them as synonyms (Harmaja 1978; 2003). The molecular phylogenetic data of Alvarado et al. (2015) reinforce the close relatedness of these genera, but *Clitocybe* and *Lepista* represent different clades. Alvarado et al. (2015) proposed four taxonomic alternatives to reconcile the taxonomic and molecular phylogenetic information, but declined to provide a definitive decision on generic circumscription. In the future, it is possible that the generic placement for *L. sordida* will change, likely to Clitocybe, but at this point that has not formally occurred. Irrespective of the final generic placement, *L. sordida* is a well-recognized distinctive species.

The fungus was first described in 1821 by Elias Magnus Fries, under the genus *Agaricus*, with the epithet *sordidus*, meaning "dirty", referring to the shades of brown that are often found on the top of the pileus, and by which it can be distinguished in the field from the similar *L. nuda* and *L. personata* (Fr.) Cooke. An overview on synonyms of *L. sordida* is given in Table 1. *Lepista sordida*, commonly called the Flesh-brown Blewit in English trivial literature, is called "Hed Chong Kho Lek" in Thailand, which means "small, violet mushroom" (Anong et al. 2008). It is characterized by a deep lilac or lilac-brown pileus color and pinkish spore print. However, the violet colors of the pileus can make it difficult to distinguish from the well-known violet, highly praised edible fungus, *L. nuda*, as well as the morphologically similar ectomycorrhizal fungus *Cortinarius violaceus* (L.) Gray. *Lepista sordida* is also known to form fairy rings that are frequently found in grasslands, however, it does not affect the vegetation (Terashima et al. 2007; Choi et al. 2010).

Chinese traditional medicine relies on many fungi which possess antitumor, antioxidant, anti-ageing, and immunomodulatory properties that are useful for a variety of therapeutic treatments (De Silva et al. 2013; Mizuno & Nishitani 2013; Thongbai et al. 2015). Recently, bioactive compounds from mushrooms such as *Cyathus pyristriatus* Thongbai, C. Richt. & M. Stadler, *Deconica* sp., *Gymnopus* sp. and *Lentinus* cf. *fasciatus* discovered in Southeast Asia were extensively studied, e.g. Thongbai et al. 2013; Surup et al. 2015; Richter et al. 2016. *Lepista sordida* has been shown to have anti-cancer, anti-microbial, and antitumor properties *in vivo* and *in vitro* from both submerged cultures and fruiting bodies. Two active diterpenoids, named lepistal and lepistol (Fig. 1) isolated from submerged cultures of the fungus induced differentiation in human leukemia cells (Mazur et al. 1996). Antimicrobial and cytotoxic-hemolytic activities were demonstrated *in vitro* on human promyelocytic leukemia (HL-60 cells) and human histiocytic lymphoma (U 937) cells (Mazur et al. 1996). Recently, Zhong et al. (2013) demonstrated significant antioxidant activity of polysaccharides from submerged cultures of *L. sordida* by oral administration in D-galactose induced aged mice. Chen et al. (2011) isolated three

new biologically active compounds from mycelial solid cultures. The lepistamides A-C and 3, 6-dioxygenated diketopiperazines (Fig. 1) showed antibacterial activity against *Staphylococcus aureus* as well as cytotoxic activity on Astc-a-1, Bel 7402, and Hela cell lines. A few studies also investigated the fruiting bodies of *L. sordida* for bioactive compounds with pharmaceutical properties (Luo et al. 2012; Miao et al. 2013). Luo et al. (2012) isolated a water-soluble polysaccharide from fruiting bodies; with a molecular weight approximately 4 $\times 10^4$ Da, by using high-performance gel permeation chromatography (HPGPC). The fraction exhibited potential immunoregulatory effects on macrophages by significantly increasing NO and TNF-α. Further studies may discover other biologically active compounds from fruiting bodies of *L. sordida*.

It has been estimated that worldwide, of approximately 650–700 edible taxa (Mortimer et al. 2012), 130 taxa could be cultivated at a commercial scale and about 22 taxa are commercially cultivated in Thailand (Boa 2004; Thawthong et al. 2014). Recent studies have reported the successful fruiting body production of novel wild strains of edible mushrooms, i.e. *Agaricus flocculosipes* R.L. Zhao, Desjardin, Guinb. & K.D. Hyde, *A. subrufescens* Peck and *Pleurotus giganteus* (Berk.) Karun. & K.D. Hyde (Klomklung et al. 2012; Luangharn et al. 2014; Thongklang et al. 2014). Over the last decade, a few studies have revealed the optimal conditions for cultivation of *L. sordida* in China (Tian et al. 2003; Li et al. 2014). However, there has been no examination of requirements for cultivation of Thai strains.

The objectives of this study were to investigate the taxonomy of this fungus through morphology and molecular similarity, to determine optimal cultivation practices for the Thai wild strains, as well as to isolate and to identify biologically active compounds from both submerged cultures and fruiting bodies of this fungus.

Materials and methods

Sample collection

Three specimens of *L. sordida* were collected from Chiang Rai and Chiang Mai provinces, Northern Thailand during the rainy seasons from June to August, 2012–2014. The specimens were hot air dried at 45 °C and kept in zip-lock plastic bags containing dehydrated silica gel as a desiccant to control humidity. All dried wild type fruiting bodies were deposited in the Herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand with the following numbers: MFLU 12–2394, MFLU 14–0042, and MFLU 15–1417; duplicates were deposited at BIOTEC Bangkok Herbarium, Thailand (BBH) under the collection numbers BBH 40573, BBH 40575 and BBH 40584, respectively.

Isolation of mycelial cultures

Pure cultures were aseptically isolated by transferring sections of internal tissue from wild fruiting bodies onto potato dextrose agar (PDA) medium containing 500mg/ml antibacterial antibiotic amoxicillin. Pure cultures were incubated at 25 °C in a dark room for 14 days. Pure culture isolates were deposited in the culture collections of Mae Fah Luang University (MFLUCC) following numbers of the corresponding cultures: MFLU 12–2394 (MFLUCC 12–0476), MFLU 14–0042 (MFLUCC 13–0898) and MFLU 15–1417 (MFLUCC 14–0769). All mention of cultures and mushrooms resulting from cultivation techniques refer to culture collection number (i.e. MFLUCC number). The cultures were maintained at 4 °C, 25 °C, and –20 °C for further studies.

Morphological characterization

Macro-morphological features were described from fresh specimens. Color codes are according to (Kornerup & Wanscher 1978). Microscopic features were studied from dried tissue mounted in H_2O and 5% aqueous KOH solution. Congo red was used for highlighting all tissues and the amyloidity of basidiospores were observed using Melzer's reagent. All microscopic features were photographed using a Nikon Eclipse 80i compound microscope fitted with a Cannon 600D digital camera. Dimensions of microscopic characters were measured using Image Frame Work (Tarosoft®, Thailand). In the description of basidiospore measurements, the following notation was used: "[*n*/*m*/*p*]" indicating *n* basidiospores were measured from *m* basidiomata of *p* collections with a minimum of 25–50 basidiospores from each basidiomata. Spore length and width are measured in side view not including the apiculus. Size and shape of basidiospores are presented in a form following the description of ranges for biometric variables according to Tulloss et al. (2005) (*a*–) *b*–*c* (–*d*), in which *b* represents the 5th percentile, *c,* the 95th percentile, while *a* and *d* are the lowest and highest extreme values measured, respectively. The range of length/width ratio of basidiospores (**Q**) is provided. In addition to Tulloss' standard format, standard deviation has been provided for **Q**' (the mean of all **Q** values computed for a single taxon).

DNA extraction, PCR and sequencing

DNA was extracted from dried specimens and mycelial cultures growing on yeast malt extract agar (YMA). For the sequencing of the dried specimens, genomic DNA extractions were performed of small pieces of dried mushroom tissue from herbarium collections using the modified CTAB (phenol-chloroform-isoamyl alcohol procedure) followed by cleaning via a silica-matrix binding procedure described in Miller (2004). DNA amplification at the University of Wyoming was performed using primers for ribosomal DNA regions (ITS4/ITS1) (White et al. 1990). Purified products were then sequenced on an ABI–3130–XL DNA Analyser (LIFE Biosystems), using the same primer combinations as for PCR at the 'Nucleic Acid Exploration Facility at the University of Wyoming. For sequencing of the mycelium, parts of it were scraped from the surface of a solid culture with a razor blade and transferred into a 0.2 ml reaction tube filled with Precellys glass beads. DNA extraction was performed using the ChargeSwitch® gDNA plant kit (Invitrogen) according to the company's protocols. PCR amplification was performed using primers developed for ITS non-protein coding regions for the primer pairs ITS4/ITS5. Purified products were cleaned by using the ChargeSwitch® PCR Clean-Up Kit (Invitrogen). Purified products were then sequenced with an ABI–3130–XL DNA Analyser (Applied Biosystems) using the same primer combinations as for PCR. Three sequences of the strains from Thailand were newly generated for this study and deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/submit/).

Optimization of culture conditions for mycelial growth

Six different media were used for optimizing mycelium growth rates, including compost extract agar (CEA), malt extract agar (MEA), oat meal agar (OMA), potato dextrose agar (PDA), Sabouraud dextrose agar (SDA) and yeast malt extract agar (YMA), all adjusted to pH 6.3. After incubation for 10 days, the growing edge of each colony from pure culture on PDA was cut out by using a cork-borer (8 mm in diameter) and placed on the center of each optimization medium in 90 mm Petri dishes. Five replicates of each medium were

incubated in a dark room, at 25 °C for 20 days. After 8 days, mycelial growth was determined by measuring the colony diameter (cm) along the plate four axes at 90° by using a ruler and calculating the average of the vertical and horizontal colony diameter (cm). Mycelial characteristics, such as color, margin and shape on the agar surface were recorded. Furthermore, the mycelial density was determined by following the scoring system of Kadiri (1998): + very scanty, 2+ scanty, 3+ moderate, 4+ abundant, 5+ very abundant. Biomass of dry mycelium was measured by melting agar media and draining away the liquid, then drying the mycelia at 30 °C for 24 hours. The weight was recorded on an electronic scale (gram). Mycelial growth and biomass were measured on days 10, 12, 14, 16, 18, 20.

Mycelial discs (8 mm) from the colony edge of 8 days old Petri dish cultures of each strain were transferred to PDA medium. The optimal temperature for mycelial growth was determined by using four different temperatures: 20, 25, 30, and 35 °C. Five replicates of each strain were incubated in a dark room. After 12 days, the growth of the colony diameter and biomass was measured as described above.

The medium exhibiting the highest growth rate and temperature optimal were used to evaluate the optimal pH. The pH was adjusted to 5, 6, 7 and 8 with hydrochloric acid (HCl) and sodium hydroxide (NaOH). The pH range of the media was measured using a digital pH-meter before autoclaving. The colony diameter and biomass for each pH was measured as described above.

Selection of the optimal substrate for spawn cultures

Different types of cereal grains and agricultural wastes were tested as spawn substrates for increasing quantities of mycelium including: mung bean, red bean, rice bran, rice husk, soy bean and sorghum, as described by Nwanze et al. (2005). All grains were cleaned and soaked in distilled water for 24 hours, re-cleaned to remove broken grains and debris, then boiled for 15–20 minutes. The grains were allowed to cool, drained to field capacity, and placed in jars and autoclaved at 121 °C for 15 minutes. After autoclaving, the substrate in all jars was gently shaken until loose, then cooled to room temperature. The mycelium growth from the edge of colonies actively growing on PDA medium was aseptically cut with an 8 mm cork-borer and transferred onto the surface of the spawn substrate. Five replicates of each inoculated spawn substrate were incubated at 25 °C until the mycelium colonized the substrate completely. The linear mycelium length was measured (cm) after 10 days and successively in a 4 days period until day 20.

Selection of compost substrate and casing layer

The compost medium was prepared with rice straw (100 kg) as the main substrate mixed with other ingredients using a modified protocol based on a previous study (Thongklang et al. 2014). The other ingredients included:, ammonium phosphate (2 kg), calcium carbonate (1 kg), calcium sulfate (3 kg), rice bran (5 kg), urea (1 kg) and a sufficient amount of water to provide 60–70% moisture. The compost substrate was pasteurized by maintaining the low temperature at 45–50 °C for 6 hours in the autoclave. The compost was allowed to cool down to room temperature before inoculating the spawn at 20 gram of colonized grain/kg compost. 5 kg of the spawn/compost substrate mixture was placed in a plastic tray (35×25×20 cm). The inoculated compost was incubated at 25 °C with relative humidity at 60–70% for the beginning of colonization. During the time taken for spawn running in the compost media, the surface was covered with plastic film to avoid drying and insect

contaminations. The substrate medium was allowed to become fully colonized before casing. A mixture of 15% sand with humus soil, pasteurized at 121 °C for 15 minutes was used as a casing material. The completely colonized compost was covered with the casing to 2.5 inch thickness and again covered with plastic. Once mycelium colonized the entire casing layer, the casing was uncovered at 25 °C and 90–98% humidity was maintained by spraying water. The number and fresh weight of fruiting bodies was recorded. The experiments were performed with five replicates of each strain in a dark room.

Statistical analysis

The optimum growth parameters and mushroom production data were subjected to statistical analysis. The mycelial growth values for growth rate, medium type, biomass, temperature and pH optimization, and spawn substrate at 20 days, were compared to obtain a mean separation using Tukey's test (p=0.05) followed by post-hoc tests. The results are expressed in a one-way ANOVA analysis using the SPSS program (Softonic International SA, Barcelona, Spain).

Investigation of the secondary metabolite production

The production of secondary metabolites in submerged cultures was examined in strain MFLUCC 14–0769 using four different media; yeast malt extract medium (YM), sugar-malt extract medium (ZM), cotton seed meal medium (Q6) and saccharose yeast malt extract medium (SYM). The mycelium was inoculated in 200 mL of each medium in 500 mL Erlenmeyer flasks at 25 °C and placed on a 120 rpm rotary shaker. After 5 days following inoculation, the free glucose was measured with glucose test strips daily until the free glucose was consumed and the pH was checked with a pH meter (method adapted from Kuhnert et al. 2014). In order to qualitively analyze the produced metabolites, an ethyl acetate extraction procedure was used on the mycelia and the submerged culture supernatant, and for the cultivated fruiting bodies (MFLUCC 14–0769), a methanol extraction procedure was used, following a protocol slightly modified from Kuhnert et al. (2014). No quantification could be performed for lack of standards. All extracts were subjected to analytical HPLC [Agilent1260 Infinity with diode array detector and C18 Acquity UPLC BEH column (2.1 × 50 mm, 1.7 µm) from Waters; solvent A: H_2O + 0.1% formic acid, solvent B: AcCN + 0.1% formic acid, gradient system: 5% B for 0.5 min increasing to 100% B in 19.5 min, maintaining 100% B for 5 min, flowrate = 0.6 mL min^{-1}, UV detection 200−600 nm] coupled to an ion trap MS (amaZon speed™, Bruker). MS- and UV-data of the compounds was compared to literature data using substance databases (CRC Dictionary of Natural Products, CAS Scifinder, Wiley-VCH Antibase).

Up-scaling and secondary metabolite isolation

For the isolation of nudic acid B, fifteen 500 ml Erlenmeyer flasks with 200 ml Q6 medium (10 g/L glycerol, 5 g/L cottonseed meal, 2.5 g/L glucose, pH set to 7.2) were inoculated with small pieces of MFLUCC 14–0769 grown on YMA. After 12 days of shaking at 140 rpm on a rotary shaker at 23 °C the free glucose was consumed. To interrupt the fermentation the mycelium was separated from the culture broth by filtration with gauze and centrifugation. For the extraction of 2.8 L supernatant, 85 g of an absorber resin (Amberlite XAD-16N) were added and incubated overnight. By adding three times 500 mL of ethyl acetate the resin was extracted on a magnetic stirrer and the organic phase was evaporated *in vacuo* at 40 °C. The remaining aquatic

phase was diluted with water and extracted three times with the same amount of ethyl acetate. After drying the ethyl acetate over sodium sulfate it was evaporated *in vacuo* at 40 °C. The fractionation of 120 mg crude extract by preparative RP-HPLC [Gilson GX270 Series HPLC system; VP 250/20 Kromasil 100 C18 ec column (Macherey–Nagel) equipped with a Kromasil 100 pre-column; acetonitrile (B)-water (A) gradient with 0.05% trifluoroacetic acid; 5 min at 25% to 45% solvent B in 30 min and 5 min at 100% B, flow rate of 30 mL/min] resulted in 3.2 mg of nudic acid B.

Results

Taxonomy and morphology

Basidiomata (Fig. 3[a-b]) small to medium-sized. ***Pileus*** 3 to 10.5 cm wide; initially convex then applanate to slightly concave, flattening out or developing a central depression at maturity, usually with a slight umbo and a wavy margin; ranging in color from deep lilac (15C8, 15D8, 15E8) to purple, turning brown from center (7D4, 7D7) when wet, hygrophanous, occasionally striate margin; context soft, thin, watery, pale lilac fading to brownish. ***Lamellae*** initially greyish lilac, light violet (15C4, 15D5) fading to buff or brown (7D4) with age sinuate or emarginate, crowded; spore print pinkish white (10A2). ***Stipe*** 2–12 × 2– 8 cm long; equal, virgate, deep lilac longitudinally striate, downy and fibrillose white at base; context solid, pale lilac fading to brownish purple. ***Annulus*** absent. ***Taste*** pleasant, slightly bitter. ***Odor*** strong fruity. ***Pileipellis*** composed of inflated hyphae, interwoven, cylindric hyphae 4–6.5 µm. ***Subhymenium*** (Fig. 3[c, f]) 22–35 µm thick; inflated cells dominating, in 3–4 layers, subglobose, ovoid, 10–20 × 8–12 µm, subtended by concatenated partially inflated hyphal segments. ***Basidia*** (Fig. 3[d-e, h-i]) 32–39(–44) × 11–13(–14) µm, narrowly clavate to clavate, mostly 4-, occasionally 2-spored, with sterigmata up to 5 µm long; clamps present at base. ***Basidiospores*** (Fig. 2[b-c]), Fig. 3[j-o]) [60/3/3] (6.0–) 6.5–7.5 (–7.85) × (3.85–) 4.10–4.60 (5.28) µm [**Q** = (1.21–) 1.29–1.58 (–1.63), **Q'** = 1.41 ± 0.10], ellipsoid, sometimes broadly ellipsoid, inamyloid, colorless, hyaline, thin-walled, finely verrucose or ornamentation cyanophilous with small apiculus.

Habitat: mixed woodland usually in areas with accumulations of decomposing leaf litter.

Distribution: widely distributed in European countries, North America and Southeast Asia.

Notes: *Lepista sordida* is a relatively common species that has been described many times previously from several different localities, for example, China, Denmark, Germany, Japan and Thailand. The morphological features of the Thai collections agree in all important aspects with previous descriptions, taking variation within species into consideration.

Molecular phylogenetic analysis

Since *L. sordida* performs to be identical species on morphological grounds, ITS sequences of *L. sordida* were subjected to a BLAST search against GenBank. The identification of this fungus was corroborated by molecular analyses. The nrITS nucleotide sequences in GenBank revealed that the most similar sequences were from *L. sordida* JN649350 from Sweden (Sjökvist et al. 2012) at 99.86 % similarity, 99% query cover; with FJ770391 from the Netherlands (Hartley et al. 2009) at 100% similarity, 99% query cover; and with KF874612 from China (Lun & Chi 2014) at 100% similarity, 96% query cover. The sequences of Thai strains of *L. sordida* MFLUCC 12–0476, MFLUCC 13–0898 and MFLUCC 14–0769 are deposited in GenBank under accession numbers KU877529, KU877530 and KU877531, respectively.

Characteristics of mycelial cultures

After 14 days of incubation in four difference media, the agar surface was fully colonized with a pale lilac mycelium. The mycelium of *L. sordida* is linear to cottony, ranging in color from white, pale lilac, purplish violet to pale grayish based on media and the number of sequential subcultures. Notably, clamp connections are always observed.

Optimal culture conditions for mycelial growth and characteristics on different media

The largest radial mycelial growth was observed on YMA. CEA and PDA were next with slightly less growth, followed by MEA and SDA. The OMA medium showed the smallest colony diameter at the end of 20 days. The color characteristics of the surface mycelium were different for the six media types. The color of mycelium growing on both PDA and YMA expressed dark purple, while it appeared purple-white on CEA and OMA. The mycelium was purple-grey on MEA, whereas, the color was purple-white at the center, pale purple towards the edges of the colony on SDA. The morphology of the colony was filamentous on all media with margin undulate on YMA, entire on CEA, OMA, PDA, SEA, and slightly eroded or lobate on MEA. The maximum yield by dry weight mycelium occurred on YMA followed by CEA, PDA, MEA, SDA and OMA. The colony diameter was similar for CEA and PDA, however, the colony density and biomass was significantly different for these two media. Notably, the fluffy or cottony surface growth of the mycelium on PDA produced less biomass than the mostly subsurface growth of CEA. The effect of six agar media on mycelial growth (cm), biomass (mg), density and color degree are given in Table 2. The characteristics on different media are shown in Fig. 4. Three *L. sordida* strains were tested for the temperature with best mycelial growth on selected YMA medium based on the maximum hyphae growth with best dry weight yield. The mycelia grew well between 25–30 °C, while 20 and 35 °C were not suitable for mycelial growth. The results of colony growth in diameter and dry weight yield at different temperatures are given in Table 3. The optimal pH for mycelial growth of *L. sordida* was in the range of pH 6–7 while pH 5 and 8 were not suitable for mycelial growth (Table 3).

Optimum substrate for spawn cultures

Mycelium started to colonize most substrates after 8 days incubation (Fig. 5). The fastest growth occurred on sorghum, followed by rice bran, red kidney bean, green bean, and rice husk. Growth on soy bean was difficult to measure as the colonies were too small to be quantified. The results indicated that sorghum is the most suitable substrate to promote mycelial growth. Effects of different spawn cultures on mycelial growth are given in Table 4.

Harvesting/production

At approximately 21 days, mycelium colonized the entire compost media and casing layer. Pre-primordia and mature fruiting bodies formed on the surface of the casing layer after 31 days at 25–30 °C. Additional flushes followed after 38–44 days and the last flush was produced at 45–52 days. Dense hyphal growth always formed on the edge of casing where fruiting bodies were produced. A continuous source of light was not important for mycelium colonization or fruiting. The successfully cultivated fruiting bodies of three Thai strains of *L. sordida* are shown in Fig. 6.

HPLC-profiling and isolation of nudic acid B

All four culture extracts showed a range of different metabolites, but neither diterpenoids (**1**) and (**2**) nor lepistamides (**3**) could be detected unambiguously. Instead several peaks with characteristic UV absorption and MS values of polyacetylenic compounds, such as 2-nonene-4, 6,8-triynoic acid or 10-hydroxydehydromatricaric acid produced by *Lepista dienii* Singer and *Clitocybe* species (Flon et al. 1958; Thaller et al. 1972), were observed. Especially one polyacetylene was detected in all extracts and was isolated from a submerged cultivation (3L) of MFLUCC 14–0769 in Q6 medium (Fig. 7). It was identified as nudic acid B, also known as diatretyne II from *Clitocybe diatreta* (Fr.) P. Kumm. (Anchel et al. 1955; Heatley et al. 1957), by NMR spectroscopy and MS spectrometric measurements. Besides having antimicrobial activities (Marx 1969) it was also reported to be toxic in mouse with a LD_{50} = 13 mg/kg (Berdy et al. 1981). In contrast, the fruiting body extracts did not contain any of these compounds but mainly fatty acids and sterols. Nudic acid B: HR-ESIMS m/z = 146.0240, calcd. for $C_8H_4NO_2$ [M+H]+ m/z = 146.0237; ESIMS m/z (rel. int.) 311 (100) $[2M+Na-H]^-$, 144 (35) $[M-H]^-$; ^{1}H-NMR (500 MHz, acetone-d6): δ = 6.76 (d, J = 16.1 Hz, 1H), 6.87 (d, J = 16.1 Hz, 1H); ^{13}C-NMR (125 MHz, acetone-d6): δ = 56.9, 66.8, 77.9, 78.7, 105.6, 121.2, 140.4, 165.5.

Discussion

This study showed that *L. sordida* collected in Northern Thailand could be domesticated and brought into cultivation successfully and therefore has a high potential for commercial production. The optimum of medium, pH, temperature and yield was investigated for the best mycelial growth. For all three strains the best growth rates were obtained using yeast malt extract agar (YMA), pH 6–8, at 25–30 °C. In general, sorghum grains were the best spawn substrate for the cultivation of *L. sordida* which showed the best mycelial growth as well as the lowest costs and good availability in Thailand. This finding is supported by the widespread use of sorghum grains for other commercially produced spawns (Ogden & Prowse 2004). Additionally, high quality spawn substrates comprised of undamaged cereal grains and proper sterilization procedures are critical for producing *L. sordida* mushrooms. The use of broken grains increased the incidence of contamination and lowered the yield (Narh et al. 2011). Strain MFLUCC 14–0769 was reproductively most successful with the highest yield of 287.5 g kg^{-1} with the most consistently well-formed basidiomata. Strain MFLUCC 12–0476 was the slowest at forming basidiomata and had the lowest yield of 93.08 g kg^{-1}. Strain MFLUCC 13–0898 produced basidiomata in every flush, with a yield of 268.3 g kg^{-1}. The colors of the basidiomata differed between these three strains. One factor that may have contributed to the color difference of the basidiomata produced was the number of times the mycelium had been sequentially sub-cultured on agar prior to inoculation onto the substrate for fruiting. The color and density of the mycelium changed markedly with each successive sub-culturing.

As mentioned in the introduction, wild strains of *L. sordida* have been domesticated previously in China by Li et al (2014), Tian et al (2003). What sets the present study apart from previous publications is a methodology whereby *L. sordida* could be cultivated by farmers and others at the lowest cost possible. Spawn for the previous two Chinese studies was produced using wheat grains. In the present study, readily available agricultural waste materials were tested as spawn substrates to make cultivation possible at a relatively low cost. Moreover, both of the previous studies used a compost bag with casing technique, whereas the present study

used a cased tray culture approach. The compost substrate in both studies relied on chicken manure mixed with agricultural waste such as dried straw and green corn straw as the main compost ingredients, while rice straw was the primary bulk ingredient in the compost in this study. All studies, including the present one, found approximately the same optimums, i.e. pH 6–7.5 at 24–28 ˚C for mycelial growth, and 20–26 ˚C with 90–98% humidity for fruiting body formation. Likewise, all studies found that certain individual strains performed better than others (i.e. faster growth, greater biological efficiency, higher quality basidiomata) under identical conditions, indicating that testing a number of strains using an optimized production process is highly beneficial.

Chemical profiles from both fruiting bodies and mycelium of this species were investigated in this study. The fruiting bodies contained mainly fatty acids and sterols, whereas, polyacetylenic metabolites such as the toxic compound nudic acid B were observed only in submerged cultivation. Therefore, *L. sordida* is a good candidate for Thai farmers with potential to cultivate an edible mushroom at commercial scale. Future research with fruiting bodies of *L. sordida* should include mating studies to increase production, selection of the best tasting strains, and best performance under standard conditions and potentially contain the highest amount of useable bioactive compounds. Developing a reliable method for cultivation reduces the need to collect the mushrooms seasonally from the wild and helps to control both quality and quantity.

Acknowledgements

Financial support by the German Academic Exchange Service (DAAD) and the Thai Royal Golden Ph.D. Jubilee-Industry program (RGJ) for a joint TRF-DAAD PPP academic exchange grant to B.T., K.D.H. and M.S. and the RGJ for a personal stipend No. Ph.D/0138/2553 in 4.S.MF/53/A.3 to B.T. is gratefully acknowledged. K.D.H. would like to thank the Thailand Research Fund for a grant (BRG5580009) to study the taxonomy, phylogeny, and biochemistry of Thai basidiomycetes. Sequences from the University of Wyoming were provided with funding from NSF grant DEB-1050292 to S.L.M. The help of Anan Thawthong and Kritsana Jatuwong at Mae Fah Luang University is grateful for carrying out mushrooms harvesting.

References

Alvarado P, Moreno G, Vizzini A, Consiglio G, Manjón JL, Setti L (2015) *Atractosporocybe*, *Leucocybe* and *Rhizocybe*: three new clitocyboid genera in the tricholomatoid clade (*Agaricales*) with notes on *Clitocybe* and *Lepista*. Mycologia 107:123–136

Anchel M (1955) Structure of diatretyne 2, an antibiotic polyacetylenic nitrile from *Clitocybe diatreta*. Science 121(3147):607–608

Anong C, Suwanarit P, Sangwanit U, Morinaga T, Nishizawa Y, Murakami Y (2008) Diversity of mushrooms and macrofungi in Thailand. 1st ed. Kasetsart, Bangkok, pp 514

Berdy J (1980) CRC Handbook of antibiotic compounds 6:371. CRC Press, Boca Raton, Florida.

Boa E (2004) Wild edible fungi, a global overview of their use and importance to people. FAO, Rome.

Chen XL, Wu M, Ti HH, Wei XY, Li TH (2011) Three new 3,6–dioxygenated diketopiperazines from the Basidiomycete *Lepista sordida*. Helv Chimica Acta 94:1426–1430

Choi JH, Fushimi K, Abe N, Tanaka H, Maeda S, Morita A, Hara M, Motohashi R, Matsunaga J, Eguchi Y, Ishigaki N, Hashizume D, Koshino H, Kawagishi H (2010) Disclosure of the "fairy" of fairy ring forming fungus *Lepista sordida.* Chembiochem 11:1373–1373

De Silva DD, Rapior S, Sudarman E, Stadler M, Xu J, Alias AS, Hyde KD (2013) Bioactive metabolites from macrofungi: Ethnopharmacology, biological activities and chemistry. Fungal Divers 62:1–40

Flon H, Anchel M (1958) Metabolic products of *Clitocybe diutreta*. II. Biochemical variation of cultures production of diatretyne 3. Arch Biochem Biophys 78:111–114

Harmaja H (1978) The division of the genus *Lepista*. Karstenia 18:49–54

Harmaja H (2003) Notes on *Clitocybe sensu lato* (Agaricales). Ann Bot Fennici 40:213–218

Hartley AJ, De Mattos-Shipley K, Collins CM, Kilaru S, Foster GD, Bailey AM (2009) Investigating pleuromutilin-producing *Clitopilus* species and related basidiomycetes. FEMS Microbiol Lett 297:24–30

Hearn MTW, Turner JL (1976) The carbon-13 nuclear magnetic resonance spectra of the antibiotic polyacetylenic nitrile, diatretyne 2 (7-cyanohept-trans-2-ene-4,6-diynoic acid), and related compounds. J Chem Soc Perkin Trans 2:1027–1029

Heatley NG, Stephenson JS (1957) Identity of nudic acid B and diatretyne II. Nature 179:1078

Kadiri M (1998) Spawn and fruit body production of *Pleurotus sajorcaju* in Abeokuta, Nigeria. Niger J Bot 11:125–131

Klomklung N, Karunarathna SC, Chukeatirote E, Hyde KD (2012) Domestication of wild strain of *Pleurotus giganteus.* Sydowia 64:39–53

Kornerup A, Wanscher JH (1978) Methuen Handbook of colour. 3rd ed. Eyre Methuen. London.

Kuhnert E, Fournier J, Peršoh D, Luangsa-ard JJ, Stadler M (2014) New *Hypoxylon* species from Martinique and new evidence on the molecular phylogeny of *Hypoxylon* based on ITS rDNA and β-tubulin data. Fungal Divers 64:181–203

Li WS, Lue YS, Wu TY, Tsai SJ, Chen MH (2014) Domestication of native *Lepista soridida* (Fr.) Singer in Taiwan J Taiwan Agric Res 63(3):216–224

Luangharn T, Karunarathna SC, Hyde KD, Chukeatirote E (2014) Optimal conditions of mycelia growth of *Laetiporus sulphureus* sensu lato. Mycology 5(4):221–227

Lun ZM, Chi YJ (2014) Optimization of extraction technique of polysaccharides from wild *Lepista sordida*. J. of Anhui Agricul Sci 2014:1–7

Luo Q, Sun Q, Wu L, Yang Z (2012) Structural characterization of an immunoregulatory polysaccharide from the fruiting bodies of *Lepista sordida.* Carbohydr Polym 88:820–824

Marx DH (1969) Influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. II. Production, identification, and biological activity of antibiotics produced by *Leucopaxillus cerealis*. Phytopathology 59:411–417

Matheny PB, Curtis JM, Hofstetter V, Aime MC, Moncalvo JM, Ge ZW, Slot JC, Ammirati JF, Baroni TJ, Bougher NL, Hughes KW, Lodge DJ, Kerrigan RW, Seidl MT, Aanen DK, DeNitis M, Daniele GM, Desjardin DE, Kropp BR, Norvell LL, Parker A, Vellinga EC, Vilgalys R, Hibbett DS (2006) Major clades of Agaricales: a multilocus phylogenetic overview. Mycologia 98:982–895

Mazur X, Becker U, Anke T, Sterner O (1996) Two new bioactive diterpenes from *Lepista sordida*. Phytochemistry 43:405–407

Miller SL (2004) New and interesting species of *Russula* from the southeastern United States 1 *Russula billsii*. Mycotaxon 89:31–38

Mizuno M, Nishitani Y (2013) Immunomodulating compounds in basidiomycetes. J Clin Biochem Nutr 52:202–207

Moncalvo JM, Vilgalys R, Redhead SA, Johnson JE, James TY, Catherine Aime M, Hofstetter V, Verduin SJ, Larsson E, Baroni TJ, Greg Thorn R, Jacobsson S, Clémençon H, Miller OK Jr (2002) One hundred and seventeen clades of euagarics. Mol Phylogenet Evol 23:357–400

Mortimer PE, Karunarathna SC, Li Q, Gui H, Yang X, Yang X, He J, Ye L, Guo J, Li H, Sysouphanthong P, Zhou D, Xu J (2012) Prized edible Asian mushrooms: ecology, conservation and sustainability. Fungal Divers 56:31–47

Narh DL, Obodai M, Baka D, Dzomeku M (2011) The efficacy of sorghum and millet grains in spawn production and carpophores formation of *Pleurotus ostreatus* (Jacq. ex Fr). Kummer Int Food Res 18:1143–1148

Nwanze PI, Khan AU, Ameh JB, Umoh VJ (2005) The effect of the interaction of various spawn grains with different culture medium on carpophore dry weights and stipe and pileus diameters of *Lentinus squarrosulus* (Mont.) singer. Afr J Biotechnol 4:615–619

Ogden A, Prowse K (2004) Spawn: how to make oyster mushroom grain spawn in a simple way. In: Gush R, editor. Mushroom growers' handbook 1: Oyster mushroom cultivation. Seoul: MushWorld–Heineart Inc, pp 62–82

Richter C, Helaly SE, Thongbai B, Hyde KD, Stadler M (2016) Pyristriatins A and B: Pyridino-cyathane antibiotics from the basidiomycete *Cyathus* cf. *striatus*. J Nat Prod 79:1684–1688

Sjökvist E, Larsson E, Eberhardt U, Ryvarden L, Larsson KH (2012) Stipitate stereoid basidiocarps have evolved multiple times. Mycologia 104(5):1046–1055

Surup F, Thongbai B, Kuhnert E, Sudarman E, Hyde KD, Stadler M (2015) Deconins A–E: Cuparenic and mevalonic or propionic acid conjugates from the basidiomycete *Deconica* sp. 471 J Nat Prod 78:934–938

Terashima Y, Fujiie A (2007) Comparison of conditions for mycelial growth of *Lepista sordida* causing fairy rings on *Zoysia matrella* turf to those on *Agrostis palustris* turf. Mycoscience 48:365–372

Thaller V, Turner JL (1972) Natural acetylenes. Part XXXV. Polyacetylenic acid and benzenoid metabolites from cultures of the fungus *Lepista diemii* Singer. J.C.S. Perkin Trans I 1972:2032–2034

Thawthong A, Karunarathna SC, Thongklang N, Chukeatirote E, Kakumyan P, Chamyuang S, Rizal L, Callac P, Hyde KD (2014) Discovering and domesticating wild tropical cultivatable mushrooms. Chiang Mai J Sci 41:731–764

Thongbai B, Rapior S, Hyde KD, Wittstein K, Stadler M (2015) *Hericium erinaceus*, an amazing medicinal mushroom. Mycol Prog 14(91):1–23

Thongbai B, Surup F, Mohr K, Kuhnert E, Hyde KD, Stadler M (2013) Gymnopalynes A and B −chloropropynyl-isocoumarin antibiotics from cultures of the basidiomycete *Gymnopus* sp. J Nat Prod 76:2141−2144

Thongklang N, Sysouphanthong P, Callac P, Hyde KD (2014) First cultivation of *Agaricus flocculosipes* and a novel Thai strain of *A. subrufescens*. Mycosphere 5:814–820

Tian GT, Feng YQ, Zhong XX (2003) A study on the domestication cultivation of *Lepista sordida.* Acta Edulis Fungi 10:52–56

Tulloss, R.E. (2005) Amaniteae: *Amanita*, *Limacella* & *Torrendia*. By Pierre Neville & Serge Poumarat, etc. [Book Review]. Mycotaxon 92: 474–484

Vizzini A, Ercole E (2012) *Paralepistopsis* gen. nov. and *Paralepista* (Basidiomycota, Agaricales). Mycotaxon 120:253–267

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds.) PCR protocols: a guide to methods and applications. Academic, New York, pp 315–322

Zhong W, Liu N, Xie Y, Zhao Y, Song X, Zhong W (2013) Antioxidant and anti–aging activities of mycelial polysaccharides from *Lepista sordida*. Int J Biol Macromol 60:355–359

Legends to Figures

Fig. 1. Chemical structures of other secondary metabolites known from submerged cultures of *Lepista sordida*

Fig. 2. Basidiospores of *Lepista sordida* by SEM. a: Four basidiospores attached to a sterigmum. b–c: verrucous basidiospore wall. Scale bar (a–c): = 1 µm.

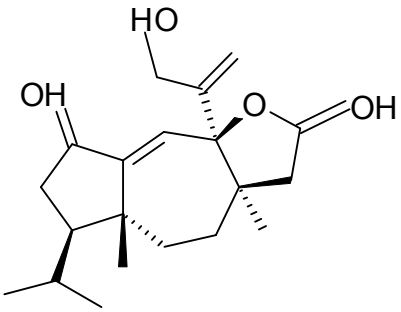
Fig. 3. *Lepista sordida* wild strain (MFLU 15–1417). a–b: Basidiomata. c–e: Basidia and subhymenium in 5% KOH. f, h–i: Basidia and subhymenium in Congo Red. g: Septate hyphae with clamp connection. j–k: Basidiospores in Congo Red. l–m: Basidiospores in Melzer's reagent. n–o: Basidiospores in 5% KOH. Scale bar (a–b) = 2 cm; (j–o) = 3 µm.

Fig. 4. Mycelial growth of *Lepista sordida* (MFLUCC 14–0769) on different agar media. a: YMA. b: OMA. c: MEA. d: PDA. e: CEA. f: SDA.

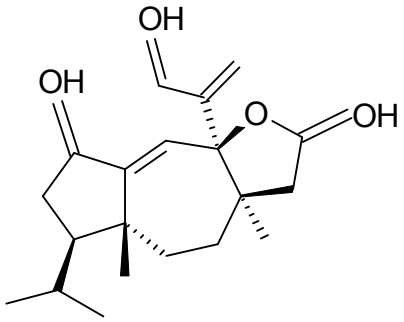
Fig. 5. Mycelial growth of *Lepista sordida* (MFLUCC 14–0769) on different spawn substrates. a: Red bean. b: Sorghum. c: Soy bean. d: Rice bran. e: Rice husk. f: Mung bean.

Fig. 6. Successful cultivation of three strains of *Lepista sordida*. a, f–g: strain MFLUCC 14–0769. b–c: strain MFLUCC 13–0898. d–e: MFLUCC 12–0476.

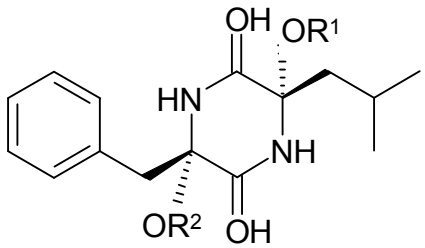
Fig. 7. Crude extract of *Lepista sordida* strain MFLUCC 14–0769 cultivated in Q6 medium. a–b: Chemical structure**,** UV/Vis and MS spectrum of the isolated metabolite nudic acid B.



(1) Lepistal



(2) Lepistol



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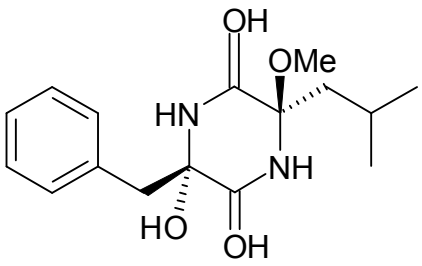
2 R^1 = R^2 = H
3 R^1 = R^2 = Me

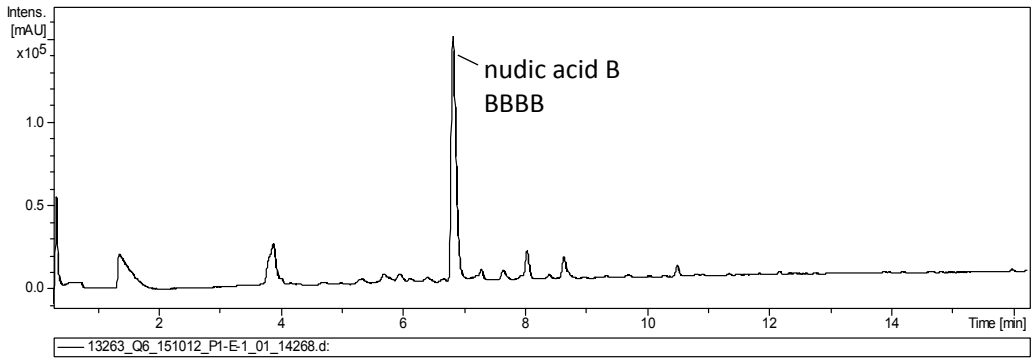
(3) Lepistamides A–C



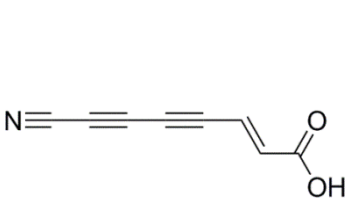
(4) Diatretol

Figs 2-6 are provided in higher quality as separate files

a)



b)



Nudic acid B

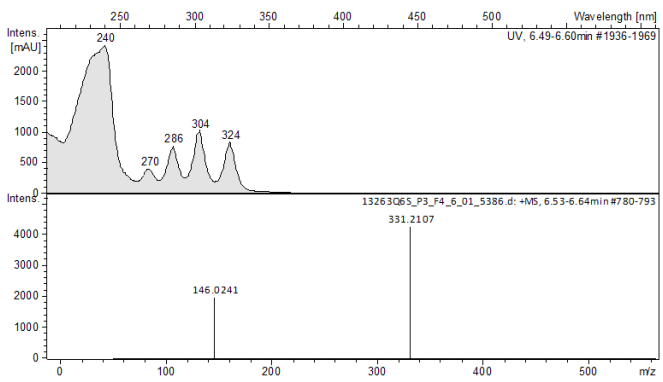


Fig. 7

Table 1 Overview on synonyms of *Lepista sordida* (For details see Index Fungorum (http://www.indexfungorum.org/) and Mycobank (http://www.mycobank.org/).

Lepista sordida (Schumach.) Singer 1951
Varieties and formae (all currently regarded as synonyms)
Lepista sordida var. *aianthina* (Bon) Bon 1980
Lepista sordida var. *calathus* (Quél.) Urbonas 1974
Lepista sordida var. *gracilis* Reichert & Aviz.-Hersh. 1959
Lepista sordida var. *ianthina* Bon 1979
Lepista sordida var. *lilacea* (Quél.) Bon 1980
Lepista sordida var. *obscurata* (Bon) Bon 1980
Lepista sordida var. *sordida* (Schumach.) Singer 1951
Lepista sordida var. *umbonata* (Bon) Bon 1980
Synonyms
Agaricus sordidus Schumach. 1803
Gyrophila nuda var. *lilacea* Quél. 1888
Gyrophila sordida (Schumach.) Quél. 1886
Lepista domestica Murrill 1915
Lepista nuda var. *sordida* (Schumach.) Maire 1916
Melanoleuca sordida (Schumach.) Murrill 1914
Rhodopaxillus sordidus (Schumach.) Maire 1913
Rhodopaxillus sordidus f. *obscuratus* Bon 1970
Rhodopaxillus sordidus f. *umbonatus* Bon 1970
Rhodopaxillus sordidus var. *aianthinus* Bon 1970
Rhodopaxillus sordidus (Schumach.) Maire 1913
Tricholoma sordidum (Schumach.) P. Kumm. 1871
Tricholoma sordidum var. *calathus* Quél.
Tricholoma sordidum var. *feuilleauboisii* Lucand & Quél. 1896
Tricholoma sordidum var. *ionidiforme* Voglino 1886
Tricholoma sordidum (Schumach.) P. Kumm. 1871

Table 2 Effect of six agar media on mycelial growth (cm), biomass (mg), density estimate, and color degree of *Lepista sordida*. Values with the same letter are not significantly different ($p < 0.05$) by the Tukey's test. Mycelial density was determined by following the scoring system of Kadiri (1998): + very scanty, 2+ scanty, 3+ moderate, 4+ abundant, 5+ very abundant

Agar media	Colony in diameter (cm)			Biomass (mg)			Density		
	Lepista sordida strains			*Lepista sordida* strains			*Lepista sordida* strains		
	MFLUCC 12–0476	MFLUCC 13–0898	MFLUCC 14–0769	MFLUCC 12–0476	MFLUCC 13–0898	MFLUCC 14–0769	MFLUCC 12–0476	MFLUCC 13–0898	MFLUCC 14–0769
CEA	5.32±0.33^{ab}	6.53±0.23^{ab}	7.20±0.10^{a}	86.33±0.76^{a}	87.12±0.21^{a}	82.67±0.76^{a}	3+	4+	4+
MEA	4.87±0.20^{c}	6.00±0.20^{bc}	6.23±0.25^{c}	44.00±0.10^{c}	48.35±0.82^{c}	50.78±0.52^{c}	3+	3+	3+
OMA	2.95±0.10^{d}	2.03±0.12^{d}	3.60±0.26^{d}	24.00±0.21^{d}	22.13±0.56^{d}	23.45±0.27^{d}	+	+	2+
PDA	5.30±0.65^{ab}	6.10±0.10^{b}	7.16±0.05a^{ab}	69.47±0.54^{b}	74.33±0.75^{b}	76.67±0.75^{b}	5+	5+	4+
SDA	3.60±0.29^{c}	3.80±0.30^{c}	5.56±0.68^{c}	42.33±0.20^{c}	44.33±0.54^{c}	46.45±0.48^{c}	3+	3+	4+
YMA	5.63±0.80^{a}	6.96±0.55^{a}	8.53±0.40^{a}	85.24±0.75^{a}	86.67±0.82^{a}	90.00±0.76^{a}	4+	4+	4+

Table 3 Effect of different temperature and pH on mycelial growth (cm) of *Lepista sordida*. Values with the same letter are not significantly different ($p < 0.05$) by the Tukey's test.

Temp. (°c)	Colony diameter			pH	Colony diameter		
	Lepista sordida strains				*Lepista sordida* strains		
	MFLUCC 12–0476	MFLUCC 13–0898	MFLUCC 14–0769		MFLUCC 12–0476	MFLUCC 13–0898	MFLUCC 14–0769
20	3.60±0.10^{c}	3.53±0.05^{c}	3.12±0.08^{c}	5	1.66±0.23^{d}	1.94±0.06^{d}	1.66±0.23^{d}
25	8.13±0.11^{a}	8.53±0.15^{a}	8.10±0.14^{a}	6	7.03±0.05^{ab}	7.50±0.12^{ab}	7.15±0.72^{ab}
30	6.46±0.05^{b}	7.63±0.11^{b}	6.93±0.11^{b}	7	7.16±0.28^{a}	7.61±0.30^{b}	7.27±0.45^{a}
35	1.18±0.17^{d}	3.25±0.13^{d}	2.97±0.43^{d}	8	2.60±0.36^{c}	2.52±0.33^{c}	2.42±0.13^{c}

Table 4 Effect of different spawn substrate on mycelia growth in diameter (cm) of *Lepista sordida*. Values with the same letter are not significantly different ($p < 0.05$) by the Tukey's test.

Spawn types	Colony diameter (cm)
Glycine max (L.) Merrill (Soybean)	0.00^{d}
Phaseolus vulgaris L. (Red kidney bean)	4.43 ± 0.05^{b}
Rice bran	4.70 ± 0.10^{b}
Rice husk	2.30 ± 0.26^{c}
Sorghum bicolor L. (Moench) (Sorghum)	5.53 ± 0.15^{a}
Vigna radiata (L.) R. Wilczek (Mung bean)	2.33 ± 0.15^{c}

Table 5 Comparison of cycle yields of harvesting of *Lepista sordida*. *Yield data = total weight of dry mushroom per kilogram of substrate.

Strain	Weight of fresh mushrooms of each flush (g)				Number of fruiting bodies	Total yield*(g kg^{-1})	Fruiting bodies after casing to latest harvest
	1st flush	2nd flush	3rd flush	4th flush			
MFLUCC 12–0476	–	150.50	296.30	18.60	113	93.08	31 days
MFLUCC 13–0898	588.32	120.30	540.25	92.17	349	268.3	52 days
MFLUCC 14–0769	800.86	550.78	–	85.77	582	287.5	48 days