FEMS Microbiology Letters 252 (2005) 223–228

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Production and characterization of Amanitin toxins from a pure culture of *Amanita exitialis*

Ping Zhang [a,*], ZuoHong Chen [a], JinSong Hu [b], BaoYang Wei [a], ZhiGuang Zhang [a], WenQi Hu [a]

[a] *College of Life Science, Hunan Normal University, Changsha 410081, PR China*
[b] *College of Life Science and Biotechnology, Nanhua University, Hengyang 421001, PR China*

Received 17 April 2005; received in revised form 23 August 2005; accepted 30 August 2005

First published online 15 September 2005

Edited by B.A. Prior

Abstract

Amanita exitialis Zhu L. Yang and T.H. Li is a lethal mushroom species recently isolated in Guangdong Province, China. In this report, a pure culture of this species was obtained for the first time. To confirm the identity of the pure culture, the internal transcribed spacer regions of the nuclear ribosomal DNA of the pure culture and of a typical fruiting body of the species were sequenced and compared. Further, amatoxins produced by pure cultures were analyzed and characterized by high-performance liquid chromatography and mass spectrometry analysis. The results showed that the pure cultures produced 728.3 ± 43.8 μg g^{-1} (dry matter) of α-Amanitin and 60.0 ± 20.7 μg g^{-1} (dry matter) of β-Amanitin, respectively, a yield which is about 10% of that produced by fruiting bodies.

Keywords: *Amanita exitialis*; Amatoxins; HPLC; ITS; MS

1. Introduction

The genus *Amanita*, one of the largest basidiomycetous genera with about 500 taxa described worldwide, contains some fatal poisonous species, such as *Amanita phalloides* (Fr.) Link, *Amanita verna* (Bull.: Fr.) Lam., *Amanita virosa* (Fr.) Bertillon, and *Amanita fuliginea* Hongo. Over 90% of the lethal cases of human mushroom poisoning have been associated with one of the poisonous species of *Amanita*. Among these species, *A. phalloides*, the death cap, is the most infamous mushroom, and has received considerable attention [1–3].

Amanita toxins produced by different *Amanita* species have been extensively studied. To date, 22 *Amanita* peptide toxins have been reported, which are classified into three main groups: amatoxins, phallotoxins and virotoxins [4]. The two major classes of toxins isolated from *A. phalloides* are amatoxins and phallotoxins. Amatoxins are by far the most dangerous toxins. The toxicity of amatoxins is due to its inhibition of RNA polymerase II, affecting protein synthesis. The toxins are preferably taken up by the liver and then circulate within the entero-hepatic circuit. Liver failure is the common cause of death by amanitin poisoning in humans [5,6]. The phallotixins specifically bind to F-actin, and thus inhibit the conversion of F-actin into G-actin. The dynamic equilibrium of F-actin/G-actin necessary for cell functions will thus be disturbed [5]. These two groups of

* Corresponding author. Tel.: +0086 0731 8872557; fax: +0086 0731 8872781.
E-mail address: zhangping0000@163.net (P. Zhang).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.08.049

cyclopeptides are widely used as research agents to inhibit RNA polymerase II and protein synthesis in biological research [7–9]. However, *Amanita* toxins are difficult to obtain since *Amanita* species are usually difficult to culture and pure cultures obtained usually either grow extremely slowly or produce little, if any, *Amanita* toxins. Thus, at present, the only practical and cost-effective way to obtain *Amanita* toxins is to extract and purify from wild carpophores collected from their natural habitat.

In March 2000, nine people in Guangdong Province of China were poisoned to death by eating poisonous wild mushrooms. The lethal mushroom was later described as *Amanita exitialis* Zhu L. Yang and T. H. Li, a newly described poisonous *Amanita* species in China [10]. Since 2000, this poisonous species has caused 20 more human deaths in southern provinces of China. Observations in the field revealed that *A. exitialis* was associated with *Castanopsis fissa* (Champ. ex Benth.) Rehd. et Wils., a higher plant only found in southern provinces of China, such as Guangdong, Yunnan and Hunan provinces. We have previously reported that this species produced a high level of toxic peptides including amatoxins and phallotoxins [11]. However, it remains unknown whether pure cultures of this species can be obtained and maintained in the laboratory and whether pure cultures also produce *Amanita* toxins.

In the present study, we report that an *A. exitialis* pure culture was successfully obtained and maintained on potato dextrose malt (PDM) medium. Sequence analysis of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA confirmed that the pure culture was generated from the fruiting bodies collected from the wild habitat. We further demonstrated that pure cultures also produce *Amanita* toxins at a yield of ~10% of that by fruiting bodies.

2. Materials and methods

2.1. Isolation and culturing of pure cultures

Fruiting bodies of *A. exitialis* were collected from Guangdong Province of China in April 2001. A primary culture was made by removing a small piece of gill from the young fruiting body with intact veil and placing it in a 18×180 mm tube containing 10 mL PDM medium (peeled potato 200 g, dextrose 15 g, $MgSO_4$ 1 g, $CaCl_2$ 0.25 g, KH_2PO_4 1 g, $(NH_4)_2HPO_4$ 0.5 g, KNO_3 1 g, thiamin–HCl 0.2 mg, malt extract 120 ml, agar 15 g to 1 L distilled water, pH 5.6). Isolates were incubated at 26 °C for 40 days. Mycelial masses from the margin of actively growing colonies were transferred to a flask containing 40 mL PDM medium. Several transfers were repeated to ensure that there was no fruiting body tissue remaining with the colonies. Microscopic characteristics of the culture were determined with a Nikon Eclipse E400 light microscope (Nikon Inc., Japan) using Congo red stain for 10 min prior to examination observation.

2.2. DNA extraction, amplification, and sequencing

Fruiting bodies of *A. exitialis* used for DNA extraction were collected at the same site, and the mycelia were incubated for 40 days as described above. Genomic DNA was then extracted from dried fruiting bodies and fresh mycelia following a modified procedures which employed $2\times$ cetyltrimetylammoniumbromide (CTAB) lysis buffer (2% CTAB, 100 mM Tris, pH 8, 20 mM Na_2EDTA, 1.4 M NaCl) [12] .

ITS regions of nuclear ribosomal DNA from both fruiting bodies and mycelia were amplified via PCR using the primers ITS4 and ITS5 [13]. PCR reactions were performed with an Eppendorf Mastercyler gradient thermal cycler (Eppendorf Inc., Germany) in 50 μL reaction mixtures containing double distilled H_2O, 1 μL DNA template, 4 μL of each 10 μM primer, 5 μL of *Taq* $10\times$ buffer, 1 μL of 10 mM dNTP mix, 3 μL of 25 mM $MgCl_2$ and 0.5 μL of 5 U/μL *Taq* polymerase. Thermal cycling parameters were as follows: initial denaturation at 94 °C for 1 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 45 s, extension at 72 °C for 50 s, and a final extension at 72 °C for 10 min. Amplified PCR products were quantified by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide and purified by microfiltration using Ultrafree-MC centrifugal columns (Millipore Inc., USA). Purified PCR products were then sequenced with primers ITS5 and ITS4 on an ABI 377 automated DNA sequencer (Perkin–Elmer Inc., USA). Reactions and programs were chosen essentially according to recommendation of the handbook. The obtained sequences were edited and aligned with DNAman (version 3.0).

2.3. Sequence analysis

ITS sequences of four related species in section *phalloideae*: *A. virosa*, *Amanita bisporigera* G. F. Atk.*A. fuliginea*, and *Amanita subjunquillea* var. *alba* Zhu L. Yang were retrieved from GenBank (GenBank Nos.: AB015676, AY550243, AY436458, DQ072729). ITS sequence of *Amanita hemibapha* (Berk. Et Broome) Sacc. (AY436460) was selected as out-group. Neighbor-joining analysis was performed with PAUP* version 4.0b10 [14], and the phylogenetic tree was obtained by running the heuristic search with tree-bisection-reconnection (TBR) branch swapping and up to 1000 random-addition sequence replications. Gaps were treated as missing data. All unambiguous characters and character-transformations were weighted equally.

2.4. Extraction of peptide toxins

Mycelia of *A. exitialis* cultured for 50 days were harvested from the surface of PDM medium and lyophilized. One gram of dried mycelia was ground in a pestle–mortar and then collected in a screw-capped bottle. Peptide toxins were extracted with 20 mL of methanol–water (1:1, v/v). The extract was incubated at 25 °C in a rotary shaker (150 rpm) for 12 h, followed by centrifugation at 8000*g* for 8 min. The supernatant was decanted and retained. To ensure the completeness of the extraction, the extraction was repeated once and the two supernatants were pooled. The extract was freeze-dried, and then re-suspended in 5 mL double distilled H_2O. Peptide toxins in the fruiting bodies were extracted similarly.

2.5. HPLC analysis

The analyses were performed using a reversed-phase HPLC method on a Waters delta 600 HPLC system (Waters Inc., USA) with a Waters 2487 variable UV detector (Waters Inc., USA). Separations were carried out on a YWG C-18 reverse phase HPLC column (300 × 4 mm I.D., particle size 10 μm, Dalian Elite Analytical Instruments Co. Ltd., China) at 40 °C. The mobile phases were (A) 0.02 M aqueous ammonium acetate-acetonitrile (90:10, v/v) and (B) 0.02 M aqueous ammonium acetate-acetonitrile (76:24, v/v). pH of solvents A and B was adjusted to 5.0 with glacial acetic acid. These were prepared with double distilled water, analytical reagent grade chemicals and HPLC grade acetonitrile. All solutions were degassed by sonication prior to use. The elution profile consisted of four isocratic steps over a total duration of 60 min: (1) 0 → 15 min, A 100% → 95%, B 0 → 5%; (2) 15 → 40 min, A 95% → 20%, B 5 → 80%; (3) 40 → 50 min, A 20% → 0, B 80 → 100%; (4) 50 → 60 min, A 0 → 100%, B 100% → 0. The mobile phase flow-rate was 1 mL/min. The absorbance of the solvent was monitored at 295 nm. Standards α-Amanitin and β-Amanitin were purchased from Sigma–Aldrich (USA). Each standard was dissolved in distilled water to concentration of 1 mg mL^{-1}. A five–point curve was obtained with injections of 3, 5, 10, 15, 25 μL. The correlation regression coefficients of both curves were greater than 0.999. α-Amanitin and β-Amanitin in the samples were identified by comparison of retention time (R_t) and co-injection with standards. Concentrations were calculated using peak areas of reference compounds. The assay repeatability was verified by assaying the same extract three times.

2.6. Mass spectrometry analysis

The compounds with the R_t close to that of α-Amanitin standard or β-Amanitin standard in the extract of the cultured mycelia were separated by HPLC, collected and purified by HPLC again under the same conditions. The purified compounds were lyophilized and re-suspended in a known volume of methanol. Molecular weight of each compound was determined using a Thermo Finnigan LCQ-Advantage MS system (Thermo Finnigan Inc., USA).

3. Results

3.1. Mycelial characters

After growing at 26 °C for 7 weeks on PDM medium, colonies with a diameter of ~35 mm appeared with white, cottony to downy and irregularly raised appearance (Fig. 1). Hyphae were thin walled without clamp connections. Younger hyphae were 2.5–4 μm in diameter (Fig. 2A), while hyphae in the older parts of the colony were frequently inflated forming subglobose vesicles (Fig. 2B).

3.2. DNA sequencing and phylogenetic analysis

A fragment (~650 bp) of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA was amplified by PCR using primer ITS4 and ITS5 from the genomic DNA isolated from pure cultures or fruiting bodies. Sequencing analysis showed that pure cultures and fruiting bodies had the same ITS (include 5.8S) sequences, demonstrating that pure cultures obtained were from *A. exitialis* fruiting bodies. The ITS DNA sequences have been deposited at GenBank under Accession No. AY855212. The result of neighbor-joining analysis based on ITS sequence data supported a clade containing four lethal *Amanita* species

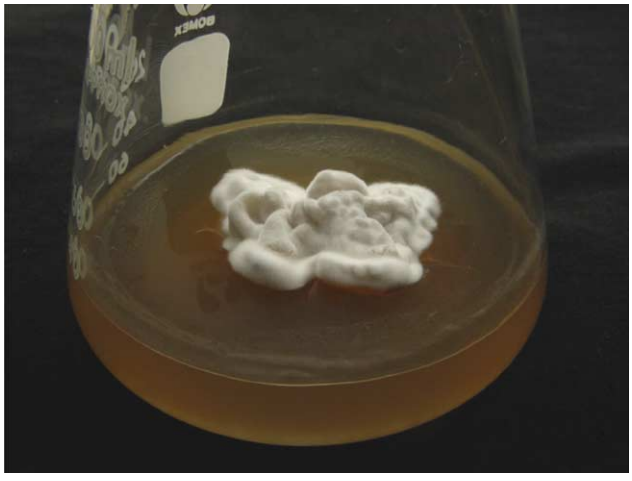

Fig. 1. A colony of *A. exitialis* incubated on PDM medium for 40 days.

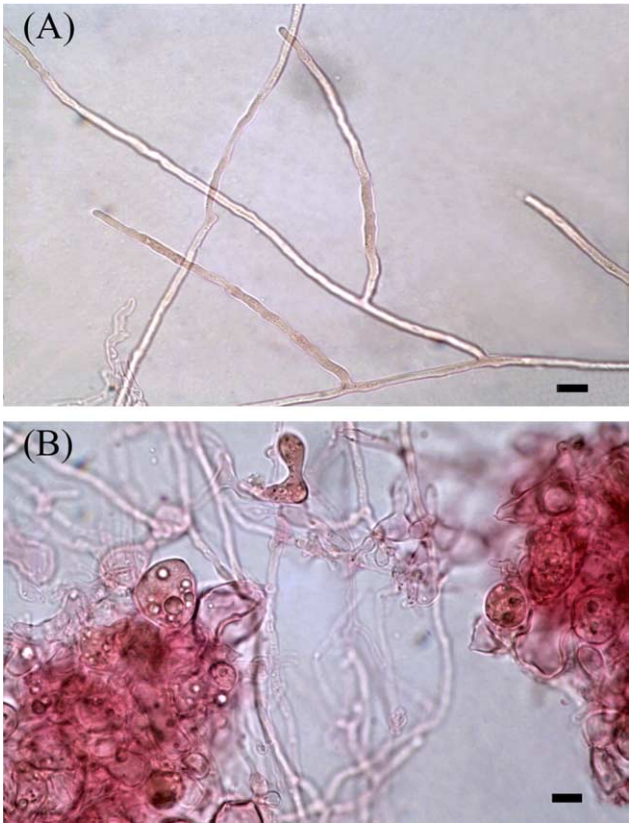

Fig. 2. Micrographs of the mycelia of *A. exitialis*: (A) filamentous mycelia from the margin of colony, (B) enlarged mycelia from the center of colony. Bar, 10 μm.

with white fruiting bodies (Fig. 3). While possessing the morphological character of 2-spored basidium *A. bisporigera*, a species localized in North America, *A. exitialis* has closer phylogenetic relationship with *A. subjunquillea* var. *alba*, a 4-spored white lethal species from China.

3.3. HPLC analysis

The retention time of α-Amanitin and β-Amanitin standard was 13.858 ± 0.573 and 8.412 ± 0.51 min, respectively. Five major peaks with the R_t of 8.815, 13.057, 14.398, 25.140, 30.557 min had been observed for extract of the fruiting bodies. Of the five major peaks observed, the peak with the R_t of 8.815 min correlated well with the standard β-Amanitin, and the peak with the R_t of 14.398 min correlated well with the standard α-Amanitin. These data suggested that these two compounds to be β-Amanitin and α-Amanitin, which is consistent with our previous report [11], Four major peaks with the R_t of 8.898, 14.307, 25.348, 31.432 min were recorded for extract from pure cultures. Of these four peaks, the compound with the R_t of 8.898 min and the compound with the R_t of 14.307 min correlated well with β-Amanitin and α-Amanitin, respectively. The HPLC chromatogram of the fruiting body partially corresponded to that of the mycelia with the exception that the peak at R_t of 13.057 min. However, the peak height of each compound from pure cultures was much lower than that in the fruiting body, suggesting that pure cultures produce amanitin toxins at a lower yield than fruiting bodies. Taken together, these results showed that pure cultures obtained in this study also produce α-Amanitin and β-Amanitin.

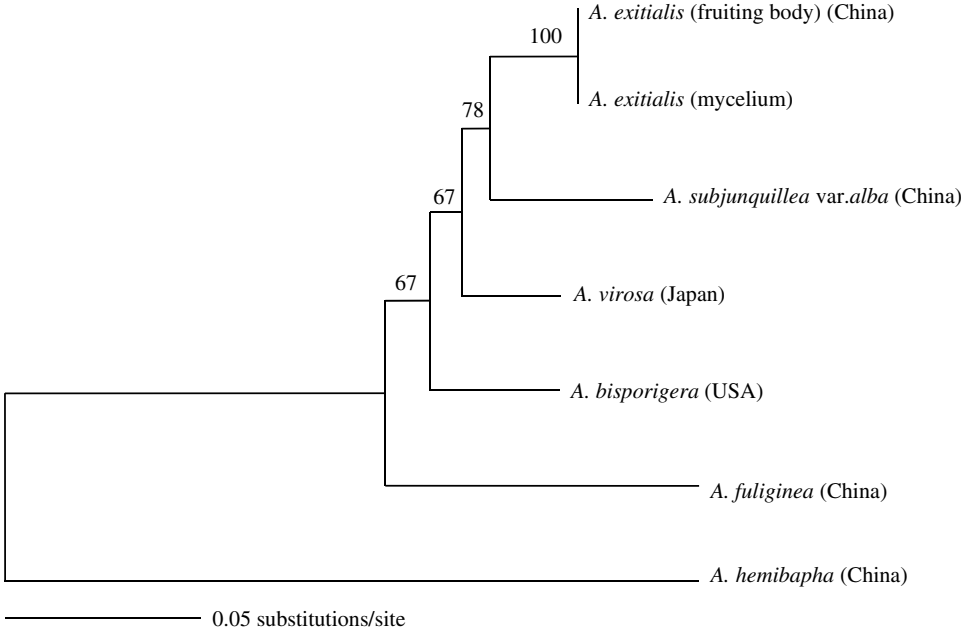

Fig. 3. Neighbor-joining tree generated for *A. exitialis* and related species based on ITS sequence data. Numbers above each internode are the percentage of 1000 bootstrap replicas supporting that binary partition (value ⩾50%). The localities of specimens are showed after the Latin names.

3.4. Mass spectrometry analysis

The results of mass spectrometry analysis showed the molecular weight of M^- ion of the compound with the R_t 8.898 min in the extract of mycelia was 918.4 Da and the molecular weight of M^- ion of the compound with the R_t 14.307 min was 917.6 Da. Thus, the molecular weight of the former compound (919.4 Da) corresponded to that of β-Amanitin, and the molecular weight of the latter compound (918.6 Da) corresponded to that of α-Amanitin. The results of mass spectrometry analysis in combination with HPLC assays determined the presence of α-Amanitin and β-Amanitin in the cultured mycelia of *A. exitialis*.

3.5. Quantifications of amatoxins in fruiting bodies and pure cultures

α-Amanitin and β-Amanitin in the fruiting body and mycelia of *A. exitialis* were quantified according to HPLC peak area. Both fruiting bodies and mycelia contained α-Amanitin and β-Amanitin. Fruiting bodies contained 6712.2 ± 496.3 and 2055.0 ± 230.9 μg g^{-1} α-Amanitin and β-Amanitin, respectively, while pure cultures contained 728.3 ± 43.8 and 60.0 ± 20.7 μg g^{-1}, respectively. It is noteworthy that although both fruiting bodies and pure cultures produce α-Amanitin and β-Amanitin, total amount of amatoxins (α-Amanitin + β-Amanitin) in fruiting bodies (8771.2 ± 727.0 μg g^{-1}) was much higher than that of pure cultures (788.3 ± 64.1 μg g^{-1}). Also it appeared that, in fruiting bodies, the β-Amanitin/α-Amanitin ratio (0.3054 ± 0.012) was higher than that in pure cultures (0.0814 ± 0.014). The content of total amatoxins in the fruiting body detected in this study was found to be a little higher than that quantified in an earlier report (8152.6 ± 1004.3 μg g^{-1}) [11], which might be due individual variations in the fruiting bodies.

4. Discussion

4.1. Culturing of Amanita

Culturing an *Amanita* species is generally a difficult task due to the fact that most species in this genus are involved in ectomycorrhizal associations with higher plants. In spite of this, over ten species of *Amanita* have been successfully cultured [15]. Some *Amanita* species such as *Amanita rubescens* and *Amanita muscaria* are relatively easy to be cultured [16–19], whereas some lethal species (e.g., *A. phalloides, A. verna* and *A. fuliginea*) are usually very difficult to be cultured due to the fact that their mycelia grow extremely slowly on media. Whether a species is easy or difficult to grow on media depends on the genetic characters of the species. The isolation techniques and the nutrition composition of the medium also strongly affect the success rate. Current available knowledge suggests that malt extract added in the medium is beneficial to the growth of the inocula. Gills from the young fruiting body with intact veil were the best source for isolation of pure cultures. Although *A. exitialis* is a lethal mushroom, our data suggested that its cultivation was relatively easier than other lethal *Amanita* species. The reasons for this phenomenon are still unknown. Average yield of the mycelia cultured on 100 mL PDM medium for 50 days approaches to 5 g (fresh weight). This is an acceptable growth rate although it is slow compared with some saprotrophic mushrooms.

4.2. Identification of the pure cultures of Amanita

According to the traditional method (Koch's Postulate), an agaric culture can be identified only when the mycelia have been cultured into the same fruiting body. However, culturing fruiting bodies of *Amanita* species on medium is almost impossible since it is difficult to mimic natural habitat where production of fruiting bodies is involved in ectomycorrhizal associations with higher plants. Besides, culturing of mycelia of most lethal *Amanita* species is also difficult. Therefore some cultural characters may be used for identification of pure cultures [17]. Unfortunately, such cultural characters are limited. In recent years, molecular techniques such as RFLP, RAPD and rDNA sequence analysis have been developed to identify pure cultures of *Amanita* species [20]. Among these techniques, ITS sequencing has been shown to be reliable and convenient [13]. In this study, the pure cultures obtained in PDM medium had the same ITS (include 5.8S) sequence as fruiting bodies. We also demonstrated that the ITS sequence of pure cultures had high similarity with those of *A. subjunquillea* var. *alba*, *A. virosa* and *A. bisporigera*, suggesting that *A. exitialis* had close phylogenetic relationship with three other white lethal *Amanita* species. For some lethal *Amanita* species, the presence of amatoxins in the mycelia can also be viewed as a biochemical evidence for the identification of pure cultures. In this report, it was shown that the pure cultures also produce the same amatoxins (α-Amanitin and β-Amanitin) as fruiting bodies. Taken together, all these data strongly suggested that the pure cultures obtained in this study were from the fruiting bodies of *A. exitialis* collected from nature habitat.

4.3. Peptide toxins in the cultured mycelia

Amanita toxic peptides have been widely used as research agents to inhibit RNA polymerase II and/or protein synthesis in biological research [7–9]. However, these toxic peptides can only be obtained from wild carpophores collected from natural habitats. Thus,

The resources to extract *Amanita* toxic peptides are limited, which is the main reason for high prices of amatoxin products. Therefore, it would greatly expend the amatoxin resources if one could extract *Amanita* peptides toxins directly from the cultured mycelia of *Amanita* species. To achieve this goal, extensive research has been carried out in recent years with limited success. There were several reports attempting to isolate and culture lethal mushroom such as *A. verna*, *A. virosa* [21] and *A. bisporigera* [22]. However, pure cultures obtained in these studies either grew extremely slowly or did not produce significant amount of toxic peptides. This study is a successful attempt of obtaining amatoxins from the cultured mycelia of an *Amanita* species, which suggests the possibility of producing these expensive reagents directly from pure cultures. Although the toxin concentration in the mycelium is about 10% of that in fruiting bodies, it is possible to increase the amatoxin production through optimizing the growth conditions. As *Amanita* toxins are produced as secondary metabolites, their production is controlled by growth conditions such as medium composition, pH and temperature, etc. Pure culture at different growth stages also may vary in the production of toxins. Besides, it is also possible to increase the amatoxin production through genetic engineering approach. In this setting, one may propose to over-express of some key enzymes which are involved in the biosynthesis of amatoxins. Attempts to elevate the concentration of the peptide toxins in the mycelia are currently under way.

Acknowledgments

This work is supported by a research Grant (No. 04C351) from Hunan Provincial Education Department awarded to P.Z. We are grateful to Dr. Dongping Li and Dr. Garry Dittberner for reviewing the manuscript and giving valuable suggestions.

References

[1] Wieland, T. and Faulstich, H. (1991) Fifty years of amanitin. Experientia 47, 1186–1193.
[2] Enjalbert, F., Gallion, C., Jehl, F., Monteil, H. and Faulstich, H. (1993) Toxin content, phallotoxin andamatoxin composition of *Amanita phalloides* tissues. Toxicon 31, 803–807.
[3] Enjalbert, F., Cassanas, G., Guinchard, G. and Chaumont, J.P. (1996) Toxin composition of *Amanita phalloides* tissues in relation to the collection site. Mycologia 88, 909–921.
[4] Wieland, T. (1983) The toxic peptides from Amanita mushrooms. Int. J. Peptide Protein Res. 22, 257–276.
[5] Wieland, T. and Faulstich, H. (1978) Amatoxins, phallotoxins, phallolysin, and antamanide: the biologically active components of poisonous Amanita mushrooms. Crit. Rev. Biochem. 5, 185–260.
[6] Cochet-Meilhac, M. and Chambon, P. (1974) Animal DNA-dependent RNA-polymerases, II. Mechanism of the inhibition of RNA-polymerases B by amatoxins. Biochim. Biophys. Acta 353, 160–184.
[7] Campadelli-Fiume, G. (1978) Amanitins in virus research. Brief Rev. Arch. Virol. 58, 1–14.
[8] Korn, E.D. (1982) Actin polymerization and its regulation by protein from nonmuscle cells. Physiol. Rev. 62, 672–737.
[9] Rebello, C.A. and Ludescher, R.D. (1998) Influence of tightly bound Mg^{2+} and Ca^{2+}, nucleotides, and phalloidin on the microsecond torsional flexibility of F-actin. Biochemistry 37, 14529–14538.
[10] Yang, Z.L. and Li, T.H. (2001) Note on three white *Amanitae* of section*Phalloideae* (*Amanitaceae*) from China. Mycotaxon 78, 439–448.
[11] Hu, J.S., Chen, Z.H., Zhang, Z.G. and Zhang, P. (2003) Analysis of the main amatoxins and phallotoxins in *Amanita exitialis* – a new species in China. Acta Microbiol. Sin. 43, 642–646.
[12] Zolan, M.E. and Pukkila, P.J. (1986) Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. 6, 195–200.
[13] White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics In: PCR Protocols a Guide to Methods and Applications (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., Eds.), pp. 315–322. Academic Press, New York.
[14] Swofford, R.E. (2003) PAUP*: Phylogenetic Analysis Using Parsimony (and other Methods), version 4.0b 10. Sinauer Associates, Sunderland, MA.
[15] Yang, Z.L., Weiß, M., Kottke, I., Oberwinkler, F., Nehls, U., Guttenberger, M. and Hampp, R. (1999) Chapter 8. *Amanita* In: Ectomycorrhizal Fungi Key Genera in Profile (Cairney, J.W.G. and Chambers, S.M., Eds.), pp. 201–230. Springer-Verlag, Berlin, 369.
[16] Antibus, R.K. and Sinsabaugh, R.L. (1993) The extraction and quantification of ergosterol from ectomycorrhizal fungi and roots. Mycorrhiza 3, 137–144.
[17] Campbell, M.P. and Petersen, R.H. (1975) Cultural characters of certain *Amanita* taxa. Mycotaxon 1, 239–258.
[18] Lamb, R.J. (1974) Effect of D-glucose on utilization of single carbon sources by ectomycorrhizal fungi. Trans. Br. Mycol. Soc. 63, 295–306.
[19] Lundeberg, G. (1970) Utilisation of various nitrogen sources, in particular bound soil nitrogen, by mycorrhizal fungi. Stud. For. Suec. 79, 1–87.
[20] Bruns, T.D., Szaro, T.M. and Gardes, M. (1998) A sequence database for the identification of ectomycorrhizal basidiomycetes. Mol. Ecol. 7, 257–272.
[21] Moser, M. (1958) Der Einfluß tiefer Temperaturen auf das Wachstum und die Lebenstätigkeit höherer Pilze mit spezieller Berücksichtigung von Mykorrhizapilzen. Sydowia 12, 386–399.
[22] Richter, D.L. and Bruhn, J.N. (1989) Revival of saprotrophic and mycorrhizal basidiomycete cultures from cold storage in sterile water. Can. J. Microbiol. 35, 1055–1060.