The Tropics is a very large area of the planet Earth with abundant and surprising biodiversity; however, it remains poorly understood. Macromycetes are no exception because they have been little studied. It is this lack of information, the desire to highlight them, and to show that these organisms should and deserve to be further studied, is what led to the publication of this book. Macromycetes are organisms with great potential for humanity as food, medicine and for biotechnological applications. This has been amply demonstrated by the few examples worldwide already studied and exploited today. Certainly, there is in tropical macromycetes an additional interest in conducting research in a poorly explored field that is necessary for better development and benefit of mankind.

In this book, specialists in the field review cultivation techniques for some tropical and subtropical species such as *Agaricus subrufescens*, *Sparassis latifolia*, *Tremella fuciformis*, *Schizophyllum commune*, *Lepista nuda*, and some promising biotechnological applications of *Auricularia* spp., *Grifola frondosa*, *Pleurotus* spp., and *Volvariella* spp. Likewise, the existence of tropical species of *Agaricus* and *Lentinula* is highlighted. Undoubtedly, this list is only a small sample of the great diversity of macromycetes present in the tropics.

The aim of this book is to draw attention to some of the research on tropical macromycetes available today. Furthermore, we sought to help motivate researchers, students, professionals and other individuals, to increase interest, and to redouble efforts to confront the enormous -Herculean- task of research that remains.

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Basic and Applied Research

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José E. Sánchez, Gerardo Mata and Daniel J. Royse
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ECOSUR

Updates on Tropical Mushrooms. Basic and Applied Research

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Editors

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Photographies of the front page: clockwise, *Agaricus subrufescens* (D.C. Zied), *Agaricus martinicensis* (C. Angelini),. *Lentinula boryana* (G. Mata*), Lepista nuda* (M.C. Bran), *Agaricus trisulphuratus* (P. Callac), *Sparassis latifolia* (Lu Ma*), L. boryana* (G. Mata), *S. latifolia* (Lu Ma). At the center, *A. subrufescens* (G. Mata)

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CONTENTS

PROLOGUE

This book contains a selection of work presented at the *International Symposium on Tropical Mushrooms*, organized by El Colegio de la Frontera Sur and Instituto de Ecología, in Tapachula, Chiapas, during 1-2 December 2016.

The Tropics is a very large area of the planet Earth with abundant and surprising biodiversity; however, it remains poorly understood. One group of organisms present in the Tropics, that have been little studied are Macromycetes. It is this lack of information, the desire to highlight them, and to show that these organisms should and deserve to be further studied, what led to the holding of this event and later, to this book.

The aim of the symposium was to bring together specialists in the field and to draw attention to some of the research on tropical macromycetes made today. Furthermore, we sought to help motivate researchers, students, professionals and other individuals involved, to increase interest, and to redouble efforts to confront the enormous -Herculean- task of research, knowledge and study remaining to be done.

From this point of view, the symposium was a great success, because researchers from around the world assembled and presented their findings and experiences on the topic. Our level of knowledge for several mushrooms was documented and also it was clearly appreciated that more research needs to be done. Macromycetes are generally organisms with great potential for humanity as food, medicine and for biotechnological applications. This has been amply demonstrated by the few examples worldwide already studied and exploited today. Certainly, there is in tropical macromycetes an additional interest in conducting research in a poorly explored field that is necessary for better development and benefit of mankind.

This book brings together a selection of the work presented at the symposium as a reliable record of what was discussed there. It is intended not only for the interest of those involved, but above all, for those who could not join us for the event.

We are especially grateful for the contributions of our collaborators for their willingness to share their knowledge and experience to complete this document. We also give special recognition and appreciation to all reviewers of the different chapters. Their disinterested and timely collaboration, their comments and suggestions were a very important and enriching input that led to the observed academic level. Last but not least, we would like to thank all persons who contributed to making this publication a reality, namely Laura López, Carla Quiroga, Margarita I. Hernández, Ana M. Galindo, Fabiola Roque, Graciela Ocampo and René H. Andrade.

The editors

INTRODUCTION

1. IMPORTANCE AND POTENTIAL OF TROPICAL MUSHROOMS

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ABSTRACT

The region between the Tropic of Cancer in the Northern Hemisphere (23.4 °N) and the Tropic of Capricorn in the Southern Hemisphere (23.4 °S) is defined as the tropics. The tropics encompass the largest range of climates and habitats on earth. Most of the ecological conditions present in the world can be found at climatic convergences of altitude and latitude in the tropics. Thus, significant areas of biodiversity are found in rainforests, dry deciduous forests, spiny forests, desert and other habitat types. Fungi have significant functions in ecosystems and are found in all kinds of environments. The many ecological and functional attributes of fungi are reflected in their great variety of forms and physiological and biochemical properties. Recent estimates of the number of fungi that exist on the earth are as many as 5.1 million species. It is estimated that fungi outnumber plants by at least 6 to 1. The significance of fungi for mankind has a long history. Fungi have been exploited in fermentation processes for thousands of years and mushrooms are known to possess pharmaceutical properties that are of major benefit to human health. It is thought that medicinal mushrooms and fungi produce over 125 medicinal functions. In the future, it is expected that additional medicinal substances and drugs may be found in, as yet, unidentified tropical mushrooms. Finally, an increasing number of edible and medicinal mushrooms of tropical origin are being commercially cultivated for food, dietary supplements and for medicine.

Keywords: edible mushrooms, macromycetes, mycology, ethnomycology, biodiversity.

TROPICS DEFINED

The tropics are defined as the region between the Tropic of Cancer in the Northern Hemisphere (23.4 N) and the Tropic of Capricorn in the Southern Hemisphere (23.4 S). The tropics cover an immense area and encompass the largest range of climates and habitats on Earth (J.M. Moncalvo 1997).

CLIMATE AND CLIMATE SYSTEMS

Climate has been defined as the statistics of weather over a 30-year interval. Several systems have been developed over the years to classify climates into similar regimes. One of the most popular systems is the Koppen classification that divides climates into five primary types labeled A through E as follows: A = tropical, B = dry, C = mild mid-latitude, D = cold mid-latitude, and E = polar. These five primary types can be further divided into secondary types such as rainforest, monsoon, tropical savanna, humid subtropical, etc. The Koppen Classification System recognizes a total of 29 climate types.

ECOLOGICAL CONDITIONS AND CLIMATE

Most of the ecological conditions present in the world can be found at climatic convergences of altitude and latitude in the tropics. These convergences have substantial ecological impacts on the types and populations of organisms found there. Thus, significant areas of biodiversity are found in rainforests, dry deciduous forests, spiny forests, desert and other habitat types.

BIODIVERSITY

Biodiversity encompasses species diversity, genetic diversity and ecosystem diversity. Fungi have significant functions in ecosystems and are found in all kinds of environments. The many ecological and functional attributes of fungi are reflected in their great variety of forms and physiological and biochemical properties. It is estimated that fungi outnumber plants by at least 6 to 1 (Blackwell 2011). Recent estimates of the number of fungi that exist on the earth are as many as 5.1 million species (Blackwell 2011). Only about 2 to 3% of these species are known. The genera of wild edible fungi found in tropical and subtropical climates are broadly similar to those found in the mycota of temperate regions (Lincoff, 2010). However, species diversity is much greater in tropical regions compared to temperate regions.

As rainforests in the tropics continue to disappear (currently at the rate of about 2,400 hectares per h) biodiversity is reduced due to extinctions. Without sufficient biodiversity on earth, survival of humankind is threatened. As the human population continues to increase, enormous pressure is placed on the planet and many species are heading toward extinction. Habitat destruction, the spread of invasive species, pollution and climate change are major threats to human survival.

As a way to help prevent extinction of species, the International Union for the Conservation of Nature and Natural Resources (IUCN) was established in 1948 and is now working in over 160 countries. This organization has had several successful interventions for reversing habitat loss and restoring ecosystems and is vital for protecting the natural resources we need to survive. IUCN's species Red List is a critical indicator of the health of the world's biodiversity. Assessments for the Red List of vertebrates, invertebrates, plants and fungi are shown in Table 1. A total of about 80,000 species have been assessed (as of 2015) but this is only half of the goal of 160,000 species by 2020. Currently, fungi are highly underrepresented (only 34 out of a goal of 14,500 have been assessed) so much more work is needed in this area. Only one cultivated species (*Pleurotus nebrodensis*) that is found in the wild in Sicily, Greece and China (Zervakis *et al.* 2014) is considered critically threatened.

Table 1. Goal and current number of species assessed for the Red List worldwide by the International Union for the Conservation of Nature and Natural Resources (IUCN) for vertebrates, invertebrates, plants and fungi (2015).

Category	Goal	Species Assessed
Vertebrates	61,635	41,517
Invertebrates	45,344	17,516
Plants	38,521	20,755
Fungi	14,500	34

HEALTH OF FORESTS

Tropical rainforests provide over 40% of the world's oxygen supply. Rainfall is critical to the health of plants, animals and fungi of the rainforest. It is generally considered that tropical mushrooms may help bring rainfall to forests. Each mushroom may release up to 30,000 spores/sec that can be carried in air currents as high as 85 km in altitude. Millions of tons of spores are released each year that may act as nuclei for the formation of raindrops in clouds. This may promote rainfall in places like the tropics that have relatively high populations of mushrooms and other fungi. Thus, spores are considered vital to productivity of tropical forests (Milliken 2015).

Mycorrhizal plants are also vitally important for the health of rainforests. Soils in over two-thirds of the world's rainforests are acidic and very low in minerals and nutrients. Fortunately, uptake of these scarce nutrients and minerals is facilitated by a unique relationship between the roots of vascular plants and fungi, i.e., mycorrhizae. Plants benefit from this relationship through the availability of additional water, minerals and nutrients supplied by the fungi while fungi benefit by additional access to carbohydrates, vital for mycelial growth, provided by the plant's roots.

ETHNOMYCOLOGY - MUSHROOMS AND HUMANKIND

Wild Mushrooms

Ethnomycology is the study of people and fungi. Wild mushrooms have been gathered for food and income for many decades in various counties. During the last 30 to 40 years, research has substantially increased our knowledge of local traditions in Africa, Asia and Mesoamerica (Mexico and Guatemala) (Boa 2004). These studies have made a distinction between mycophilia and mycophobia. In mycophillic societies, fungi are esteemed and there is a long tradition of popular use. Mycophobic cultures have a minor regard for mushrooms and they are often actively feared (Boa 2004). China, Japan and the Koreas are examples of mycophyllic societies while India, Pakistan and other former British colonies such as Australia and the U.S.A. are usually considered mycophobic. Traditions vary within countries, too. For example, Hispanic Mexico is generally considered mycophobic while native peoples in Mexico are considered mycophilic (Lincoff 2010). Another example is Africa, where most of the continent is mycophobic with some scattered peoples in West and East Africa considered mycophilic. Variable traditions also exist in Tanzania (Boa 2004).

Wild mushrooms are collected and used as food and to generate income in over 80 countries. There is a relatively large diversity of different types (1,000-plus species) recorded for these purposes. Some of the wild mushrooms are exported but most are used for subsistence. In 2013, wild mushrooms represented about 8% of the component value of the world mushroom industry (Fig 1) (Royse *et al.* 2017).

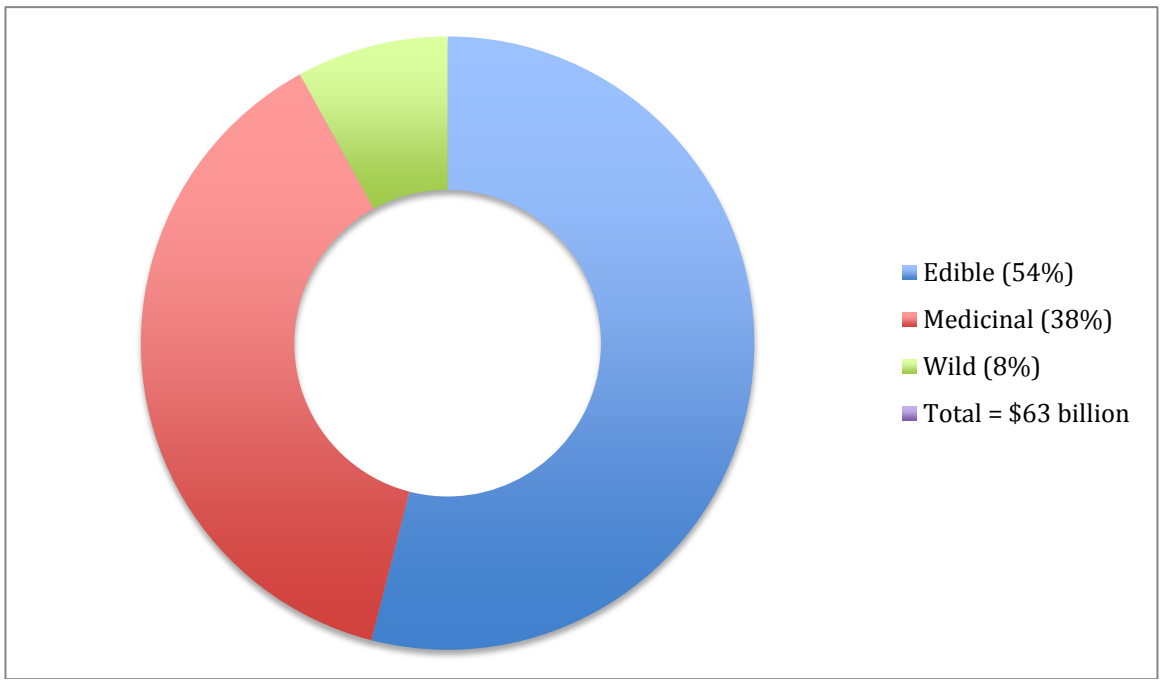

Figure 1. Component value of the world mushroom industry in 2013 showing wild mushrooms accounting for approximately 8% or $5.04 billion of the total value ($63 billion).

Medicinal mushrooms

Medicinal mushrooms are routinely used in traditional Chinese medicine and wild medicinal mushrooms are also used in Mexico and many other countries. It is thought that medicinal mushrooms and fungi produce over 125 medicinal functions (Chang and Wasser 2012). These include antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, anti-hypercholesterolemia, antiviral, antibacterial, antiparasitic antifungal, hepatoprotective, detoxification and anti-diabetic. Many, if not all basidiomycetes contain biologically active polysaccharides in fruitbodies and cultured mycelium and broth (Chang and Wasser 2012). Component value of medicinal mushrooms for the world in 2013 was estimated at $23.9 billion or about 38% of the total value (Fig. 1) (Royse *et al*. 2017).

One medicinal component of mushrooms that has received considerable attention in recent years is ergothioneine (ERGO) (Dubost *et al*. 2006). This compound functions as an intra-mitochondrial antioxidant that acts to interrupt the vicious cycle of oxidative stress, cell injury and inflammation. ERGO is made in relatively few organisms with fungi as one of the main sources (Kalaras *et al*. 2017). Humans acquire ERGO through diet and it accumulates in erythrocytes, bone marrow, liver, kidneys, seminal fluid, and the eyes. Humans have a specific transporter gene (OCTN1) so it is thought that ERGO plays an important role as a "cytoprotectant" in human health (Grundemann 2012). Some scientists have proposed that ERGO be elevated to vitamin status (Paul and Synder 2010).

It is estimated that nearly 90% of human diseases known to medical science can be treated with prescription drugs derived from nature (Torrence 2013). In the future, it is expected that additional medicinal substances and drugs may be found in, as yet, unidentified tropical mushrooms and in those that

are already known. The potential of these fungi for improving human health is enormous and their potential impact is incalculable.

MUSROOM SAFETY

Some mushrooms, harvested from the wild, are known to be poisonous. Fungal poisoning is a relatively rare event and is related to local habits, economic factors and lifestyle. Among thousands of known mushroom species, fewer than a hundred are toxic. For example, *Amanita phalloides* and several other members of the genus *Amanita*, as well as *Conocybe*, *Galerina* and *Lepiota* contain amatoxins that can cause severe gastroenteritis and hepatic necrosis. Ingestion of *Psilocybe semilanceata* (liberty cap), containing a potent halluciogen, may cause anxiety, panic reactions, and peripheral sympathomemetic symptoms (Fenney *et al*. 2014). Another toxin, contained in *Cortinarius speciosissimus*, may cause permanent kidney failure. Most mushroom poisonings occur due to ingestion of a toxic mushroom misidentified as an edible one. It is always best to have the wild mushroom inspected by a certified expert or mycologist prior to consumption (Schenk-Jaeger *et al*. 2012).

SUMMARY

The tropics cover an immense area and encompass the largest range of climates and habitats on earth. Fungi outnumber plants by at least 6 to 1 and a very small percentage of the 5.1 million species of fungi on the earth are probably known. Tropical mushrooms are invaluable today – serving as a source of food and income and helping to maintain the health of forests and of humans. It is expected they will be even more valuable to humankind in the future.

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Biodiversity and Taxonomy

2. TROPICAL SPECIES OF *AGARICUS*

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ABSTRACT

Agaricus is a large genus of mushrooms including species of nutritional and medicinal interest. With research programs developed over the last decade, a larger diversity of *Agaricus* came to light. The present review focuses on tropical species of *Agaricus* and their classification, including a discussion of the definition of tropical species, a brief history of the taxonomic system of classification since 2000, an updated presentation of the revised system including its recent emendation, and the distribution of tropical species in the subgenera and sections of the current system. Tropical species of interest and methods to identify tropical species of *Agaricus* are also introduced. The new classification system should facilitate studies of tropical *Agaricus;* however, more investigations in unexplored areas are necessary to complete the classification system and to clarify the evolutionary history of the genus in which climate and geography seem to have been the main factors of diversification.

Keywords: classification, climate, fungal diversity, subgenus, systematics, taxonomy

THE GENUS *AGARICUS*

The genus *Agaricus* (Agaricales, Basidiomycota) is characterized by a stipe separable from the pileus (heterogeneous context) provided with one or several annuli and free lamellae that produce brown basidiospores. Its distribution range extends to all continents except Antarctica (Parra 2008, Zhao *et al.* 2011). Species of *Agaricus* are saprobic and grow in forest, grassland, dune, or any place with decaying organic matter (Karunarathna *et al.* 2016). Zhao *et al*. (2011) counted 386 recognized species including 183 that were tropical. With 170 new species described from 2011 to September 2018, the number of species recognized today exceeds 500 (Chen *et al*. 2017, Karunarathna *et al*. 2016, Kerrigan 2016). In addition, many putative new species have not yet been named and species diversity remains poorly known in many regions. Indeed, 185 new species that were proposed and included in phylogenetic analyses since 2000 (see list of species in Appendix 2, at the end of the book) are heterogeneously distributed as follows:

--- 55% (102) were described from Asia, mostly from China and Thailand (Ariyawansa *et al*. 2015, Chen *et al*. 2012, 2015, 2017, Dai *et al*. 2016, Gui *et al.* 2015, He & Zhao 2015, He *et al.* 2017, 2018a,b, Hyde *et al*. 2017, Karunarathna *et al*. 2014, Li *et al*. 2014, 2016, Liu *et al*. 2015, Thongklang *et al*. 2014a, 2016, Wang *et al*. 2015, Zhang *et al.* 2017, Zhao *et al*. 2012a, b, 2016, Zhou *et al*. 2016), and some from India, Iran, and Pakistan (Bashir *et al.* 2018, Chen *et al*. 2016c, Kaur *et al.* 2016, Mahdizadeh *et al.* 2018).
--- 25% (47) were from the Americas, mainly from North America (Callac & Mata 2004, Kerrigan 2016, Kerrigan *et al.* 2008), and some from the Caribbean and South America (Albertó *et al.* 2000, de Meijer 2008, Drewinski *et al.* 2017, Parra *et al.* 2018).
--- 14% (26) were from Europe (Blanco-Dios 2001, Callac & Guinberteau 2005, Kerrigan *et al*. 2008, Lanconelli 2002, Mahdizadeh *et al.* 2018, Mua *et al.* 2017, Parra 2013, Parra & Arrilaga 2002, Parra & Caballero 2017, Parra *et al.* 2011, 2014, 2015),
--- 5% (9) were from Oceania (Geml *et al*. 2007, Lebel 2013, Lebel & Syme 2012),
--- 1% (1) was from Africa (Hama *et al*. 2010).

We excluded *A. dilutibrunneus* R.L. Zhao (Zhao *et al.* 2016) from this count because it is a doubtful species of which the sequences of different genes seem mutually incompatible (Mahdizadeh *et al*. 2018). Among the 185 new species proposed since 2000, *A. heinemannii* Albertó & G. Moreno (Albertó *et al.* 2000), *A. pachydermus* T. Lebel (Lebel & Syme 2012), *A. patialensis* M. Kaur & Harw. Kaur (Kaur *et al.* 2016), and *A. stijvei* de Meijer (Meijer 2008) are the only species for which sequence data are not available.

Species of *Agaricus* have both nutritional and medicinal interest. Certain edible species are tasty and frequently collected in the wild for consumption, such as *A. augustus* Fr. and *A. campestris* L. : Fr., but the latter has never been successfully domesticated (Grigson 1975). In contrast, *A. bisporus* (J.E. Lange) Imbach (the button mushroom) is not encountered frequently in the wild but has been domesticated and remains the species that is the most cultivated worldwide (Chang 1999, Savoie *et al*. 2013). Some other species are cultivated to a lesser extent, such as *A. bitorquis* (Quél.) Sacc. and *A. arvensis* Schaeff. *Agaricus subrufescens* Peck (the almond mushroom) is cultivated primarily for medicinal use. Despite their ecological, nutritional, medicinal, and economical potential interest, species of *Agaricus* remain little documented in certain countries, particularly in the tropics.

Breeding work, developed mainly for *A. bisporus*, requires genetic resources and knowledge of its reproductive biology and genetics. For this purpose, a large collection of germplasm is currently available (Callac *et al*. 2002, Savoie *et al*. 2013); the three main types of life cycles (heterothallism, pseudohomothallism, or homothallism) are present in this species (Callac *et al*. 2003, Savoie *et al*. 2013) and complete genomes and genetic maps are available (Foulongne-Oriol *et al*. 2010, Morin *et al*. 2012). A wide diversity of germplasm, life cycles, and a genetic map are also documented in A. *subrufescens* (Foulongne-Oriol *et al*. 2016, Rocha de Brito *et al*. 2016, Thongklang *et al*. 2014b).

We focus, with the present review, on the tropical species of *Agaricus* and their classification. Despite efforts of some mycologists regarding molecular delimitation of species, we are a long way from fully clarifying the classification of *Agaricus*. The state-of-the-art of classification that we detail here most likely will be considerably improved in the coming years. This new classification was required to accommodate the numerous clades of tropical species evidenced in Zhao *et al.* (2011).

WHAT DOES IT MEAN TROPICAL SPECIES OF *AGARICUS*?

Our definition of tropical *Agaricus* is empirical and neither absolute nor definitive. Among several hundred samples that were collected during ten years in north Thailand (including highlands), none of the species known in Europe was detected except in two specific cases: (1) *A. subrufescens*, which is one of the rare cosmopolitan species of *Agaricus* (Wisitrassameewong *et al*. 2012) and (2) *A. endoxanthus* Berk. & Broome, which is a tropical species relatively frequently introduced in tropical greenhouses in temperate areas (Chen *et al*. 2016c, Zhao *et al.* 2012a). Interestingly, 500 km to the north, in the Yunnan Province of China, it is possible to find both 'temperate species' including certain known in Europe such as *A. abruptibulbus* Peck and 'tropical species' known in north Thailand such as *A. flocculosipes* R.L. Zhao, Desjardin, Guinb. & K.D. Hyde (Zhao *et al*. 2016). This boundary between south China and north Thailand that temperate species cannot cross corresponds to the climatic boundary between humid subtropical (Cfa or Cwa) and tropical (A) climate areas according to the Köppen-Geiger climate classification (Peel *et al*. 2007). We have simply defined the tropical species as the species native from the tropical climate regions.

Are such species exclusively tropical? It is generally the case but there are exceptions as *A. endoxanthus* and *A. subrufescens* cited above. The latter species is cosmopolitan as, to a lesser extent, *A. bisporus*

reported from D. R. Congo (Heinemann 1956) and possibly *A. bitorquis*. There are also some tropical species such as *A. flocculosipes* that extends to neighboring subtropical climatic areas. Possibly, some species discovered first in subtropical regions could be in fact tropical species colonizing in subtropical areas. In other respects, *Agaricus* species in relatively arid/hot climate (including hot Mediterranean or hot temperate) are poorly known because the fruiting periods are often short and unpredictable.

Tropical and non-tropical species are generally grouped in distinct clades that diverged a long time ago (Zhao *et al*. 2016). This suggests that adaptation to a different climate is not a frequent event. For temperate species, fruiting at high temperature (i.e. 25°C or more) is not an optimal condition because the hot season is not sufficiently rainy for optimal reproductive fitness. The rare cosmopolitan species of *Agaricus* belong to clades that are not strictly tropical or temperate. Their ancestral condition is unknown. Investigations of genetics of fruiting ability at high temperature in *A. bisporus* are in progress (Foulongne-Oriol *et al*. 2014, Navarro *et al.* 2014). Current cultivars of this species are unable to fruit at 25°C. However, fruiting tests revealed that the percentage of wild isolates able to fruit in cultivation at 25°C was 100% with a good yield for the population of *A. bisporus* var. *burnettii* Kerrigan & Callac distributed in the Sonoran Desert of California. On the contrary, it varies, on average, from 35% to 78% with a lower yield among different populations of *A. bisporus* var. *bisporus* from temperate regions of Europe and North America (Largeteau *et al*. 2011). These data suggest that temperate populations of this species retain an evolutionary potential to adapt to a hot climate. Unfortunately, it was not possible to study the adaptation to tropical climate in absence of an isolate from the only known population in D. R. Congo. However, we suspect that optimal fruiting temperature is not the single criterion for adaptation to tropical or temperate climates.

We also use the terms of 'tropical section' or 'tropical subgenus' to indicate that most species belonging to the corresponding clades are known from tropical climate regions and none are known from temperate climate regions. We could not use a strict definition because there are always some species that can be found in neighboring subtropical regions (as in the palaeotropical *A.* sect. *Brunneopicti*) or even hot temperate climate (as in the neotropical *A.* subg. *Minoriopsis*), but never in typical temperate regions. In other respects, the terms palaeotropical and neotropical are used in the strict sense at any taxonomic rank. They indicate that all specimens of the taxon are exclusively known today from Africa or Asia, or from the Americas, respectively. The geographical status of palaeo- or neotropical clades may change in the future with reports of new specimens or species.

A BRIEF HISTORY OF THE CLASSIFICATION SINCE 2000

Until the year 2000, taxonomic classification did not reflect phylogeny of the species, mainly because few morphological characters were available and their evolutionary histories were completely unknown. For example, species were grouped in sections according to their discoloration (pink, red, yellow, or none) when the mushroom is rubbed or cut. However, this criterion of flesh discoloration was overemphasized because it was subsequently shown that unrelated sections (*Arvenses* and *Xanthodermatei*) are both characterized by yellow discoloration. Morphology of the annulus, macrochemical reactions and organoleptic characters seems more reliable but in quasi-absence of knowledge on their function and their adaptive role, and more generally on the ecology and physiology of the species it remains very difficult to apprehend their evolutionary history. However, before the development of molecular tools, some mycologists began to emphasize other criteria such as the structure of the annulus and others suspected that some secotioid genera should be included in *Agaricus* hypothesizing that their gasteroid appearance would result from adaptation to dryness. Despite the attempts of mycologist Paul Heinemann to propose new subgenera and sections for tropical species, many of them were classified in sections based on temperate species.

From 2000 to 2010 classification continued to progress. Phylogenetic reconstruction of sections *Bivelares* (including the tasty edible *A. bisporus*) and *Xanthodermatei* (including toxic species such as *A. xanthodermus*) were performed based on rDNA-ITS sequences (Challen *et al*. 2003, Kerrigan *et al*. 2005, 2008). Analyses using ITS and LSU sequence data (Geml *et al*. 2004, Mitchell & Bresinsky 1999) also indicated that these two sections were unexpectedly closely related to each other while they exhibit different flesh discolorations (pink and yellow, respectively). At that time, eight temperate sections were considered: *Agaricus*, *Arvenses*, *Bivelares*, *Chitonioides*, *Minores*, *Sanguinolenti*, *Spissicaules* and *Xanthodermatei*. Although sections *Xanthodermatei* and *Arvenses* exhibit a yellow discoloration, they are not phylogenetically related. Several secotioid species were included in the genus and 13 new species were described. Structure of the annulus (superous vs. inferous; simple vs. double or two layered, microscopic features), odor, and cross reaction of Schäffer (aniline × nitrogen acid) became major criteria of classification.

Monographs using molecular data for the first time, were completed on European species (Parra 2008, 2013) and North American species (Kerrigan, 2016). Concomitantly, species from Asia (mainly Thailand and China) were studied through a collaboration between European and Asian teams (mainly P. Callac at INRA-MyscSA in France and K. D. Hyde at MFLU in Thailand). Following field work of R-L Zhao that began in 2005 in Thailand, these studies were supported in 2010 by an Integration Research Grant from the European Distributed Institute of Taxonomy (EDIT). They included European specimens of reference and many tropical species, mainly from Thailand and to a lesser extent from Africa and the Caribbean thanks to collaboration with the University of Lille, France (R. Courtecuisse, LIP Herbarium), the National Botanic Garden of Belgium (O. Raspé and A. De Kesel, BR Herbarium) and L. A. Parra (private herbaria LAPAG, LAPAM and LAPAF; Avda. Padre Claret n°7, 5°G, 09400 Aranda de Duero, Burgos, Spain). From 2011 to September 2018, 170 new species were described, some subsections and sections were proposed (Parra 2013, Kerrigan 2016), numerous tropical clades were delineated (Zhao *et al*. 2011), a revised system of classification was proposed (Zhao *et al*. 2016) and emended (Chen *et al*. 2017, Parra *et al*. 2018, He *et al*. 2018a). Although all these studies contributed more or less directly to the revision of the classification, we will focus here on the first work that included tropical species in phylogenetic analyses (Zhao *et al*. 2011) and on the studies supporting tropical subgenera or sections (Chen *et al*. 2015, 2017, Parra *et al*. 2018, Zhao *et al*. 2016, He *et al*. 2018a).

WHY WAS A REVISED CLASSIFICATION REQUIRED?

There were three main reasons for revising the classification. The first reason was the small number of subdivisions of the genus. Indeed, eight sections were not sufficient for a number of species that already exceeded 500 and will continue to increase. The second reason was that three of the traditional sections (*Sanguinolenti*, *Spissicaules*, and *Xanthodermatei*) were polyphyletic and required splitting into several sections:

--- three sections (*Bohusia*, *Nigrobrunescentes*, and *Sanguinolenti*) for *A.* sect. *Sanguinolenti* (Parra 2008, Parra *et al*. 2014, 2015, Peterson *et al*. 2000, Zhao *et al*. 2011, 2016),

--- three sections (*Rarolentes*, *Spissicaules*, *Subrutilescentes*) for *A.* sect. *Spissicaules* (Kerrigan 2016, Zhao *et al*. 2011, 2016)

--- two sections (*Hondenses*, *Xanthodermatei*) for *A.* sect. *Xanthodermatei* (Kerrigan 2016, Kerrigan *et al*. 2005, Thongklang *et al*. 2014a, Zhao *et al*. 2011, 2016)

With these five new sections, the number of sections increased from eight to 13 for species distributed in temperate climate regions.

The third reason for revising the classification was to accommodate unclassified tropical clades described by Zhao *et al*. (2011). In this study, there were seven strongly supported tropical clades (TRI to TRVII)

and four lesser supported tropical clades (TRa to TRd). In the revised system of classification proposed by Zhao *et al*. (2016) and subsequently emended (Chen *et al.* 2017, Parra *et al.* 2018, He *et al.* 2018a), one tropical subgenus and 11 tropical sections were retained. Correspondence between the clades observed by Zhao *et al*. (2011) in their phylogenetic tree, based only on ITS sequence data and the 6 subgenera and 24 sections of the revised system based on multi-gene phylogenetic analyses with dated trees (Zhao *et al.* 2016, Chen *et al.* 2017, He *et al.* 2018a) and taxonomic emendations (Parra *et al.* 2018), are shown in Table 1.

Table 1. Distribution of tropical species in the current revised system of classification*.

Revised system			Major clades (Zhao *et al.* 2011)
Study	Subgenus	Section	
Zhao *et al.* 2016 He *et al.* 2018a	*Pseudochitonia*	***Flocculenti***	Closely related to TRa
		Brunneopicti	TRI
		Trisulphurati	TRb (in part)
		Crassispori	TRb (in part)
		Cymbiformes	TRb (in part)
		Rubricosi	TRc
		Bivelares	Bivelares
		Chitonioides	Chitonioides
		Xanthodermatei	Xanthodermatei (in part)
		Hondenses	Xanthodermatei (in part)
		Sanguinolenti	Sanguinolenti I
		Bohusia	Sanguinolenti II
		Nigrobrunnescentes	Sanguinolenti III
	Agaricus	*Agaricus*	Agaricus
	Spissicaules	***Amoeni***	TRIII
		Rarolentes	Spissicaules (in part)
		Spissicaules	Spissicaules (in part)
		Subrutilescentes	Spissicaules (in part)
	Flavoagaricus	*Arvenses*	Arvenses
Chen *et al.* 2017 Parra *et al.* 2018	*Minoriopsis*	***Minoriopsis***	TRd (in part)
		Kerrigania	TRII
	Minores	***Leucocarpi***	
		Pantropicales	
		Minores	TRV, TRVI, TRVII, Minores
	Unclassified		TRIV (*A. deserticola*)

*Sections are indicated in red, bold red or highlighted when the corresponding clade includes a proportion of tropical species reaching 10-20%, reaching or exceeding 40-50%, or of 100%, respectively. Highlighting in green, blue, or yellow, indicates that the clade exclusively includes to our knowledge either palaeotropical, neotropical species, or both palaeo- and neotropical species, respectively.

Figure 1. a. *Agaricus niveogranulatus*, LD2012148 (*A.* sect. *Brunneopicti*), b. *Agaricus suthepensis*, LD2012100 (*A.* sect. *Amoeni*), c. *Agaricus trisulphuratus* complex, LD2012176 (*A.* sect. *Trisulphurati*), d. *Agaricus variicystis*, LD201228 (*A.* sect. *Crassispori*).

DISTRIBUTION OF TROPICAL TAXA IN THE REVISED SYSTEM OF CLASSIFICATION

Zhao *et al*. (2016) proposed a revision of the taxonomic system of classification in the genus *Agaricus* considering stem age as a criterion for standardizing taxonomic ranks (Avise & Johns 1999). In their Maximum Clade Credibility tree of *Agaricus* based on ITS, LSU, *tef1α*, and *rpb2* genes, strongly supported clades that diverged about 30-33 million years ago (Ma) or about 18-26 Ma were ranked as subgenera or sections, respectively. Zhao *et al.* (2016) proposed five subgenera, then a sixth subgenus was added by Chen *et al*. (2017). After a taxonomic emendation (Parra *et al.* 2018) and a recent contribution (He *et al.* 2018a), the current system includes 24 sections (Table 1; note that the species listed in Appendix 2 are grouped by section).

Eleven of the 24 sections are tropical and their history is briefly presented below with species illustrated in Figs. 1 and 2 for eight of them.

--- Two sections (*Brunneopicti* and *Trisulphurati*) have been previously proposed by Heinemann (1956). However, the current concept of *A.* sect. *Brunneopicti*, that corresponds to clade TRI, is much broader than its original circumscription (Chen *et al.* 2015, Karunarathna *et al.* 2014, Zhao *et al.* 2016). This section is illustrated in Fig. 1a with *A. niveogranulatus* Linda J. Chen, R.L. Zhao, Callac & K.D. Hyde showing the pileus and stipe base covered with granulose or punctiform squamules. In *A.* sect. *Trisulphurati*, sporocarps of the species of the *A. trisulphuratus* Berk. complex exhibit an unusual orange color (Fig. 1c). These two sections are palaeotropical and belong to *A.* subg. *Pseudochitonia*.

--- Four sections (*Amoeni*, *Crassispori*, *Flocculenti*, and *Rubricosi*) were recently proposed by Zhao *et al.* (2016). *Agaricus* sect. *Amoeni* is a pantropical section in *A.* subg. *Spissicaules*. *Agaricus suthepensis* Linda J. Chen, K.D. Hyde & R.L. Zhao, illustrated in Fig. 1b, should be sequenced for a reliable identification. The three remaining sections are in *A.* subg. *Pseudochitonia*. *Agaricus* sect. *Rubricosi*, which is pantropical, is illustrated (Fig. 2c) with a specimen from Martinique of the species *A. fiardianus* Callac & L.A. Parra (≡ *A. magnivelaris* Pegler; Mahdizadeh *et al.* 2018). This species is better characterized by its narrow spores than by a large annulus according the notes of J.-P. Fiard. The two remaining sections (*Crassispori*, and *Flocculenti*) are palaeotropical and are illustrated by *A. variicystis* Linda J. Chen, K.D. Hyde & R.L. Zhao (Fig. 1d) characterized by lageniform cheilocystidia, and *A. erectosquamosus* Linda J. Chen, K.D. Hyde & R.L. Zhao (Fig. 2a) characterized by erect, dark brown squamules on the pileus, respectively.

--- Two sections (*Leucocarpi* and *Pantropicales*) belong to *A.* subg. *Minores*. *Agaricus* sect. *Leucocarpi* was recently proposed by Chen *et al.* (2017) for a single Asian species, *A. leucocarpus* Linda J. Chen, Callac, R.L. Zhao & K.D. Hyde (Fig. 2b). This species exhibits a Schäffer positive reaction (Parra *et al.* 2018) contrarily to what was indicated in its original description. In a recent study, at least one unnamed species from the Caribbean clusters with *A. leucocarpus* in *A.* sect. *Leucocarpi* (Ortiz-Santana *et al.* 2018), which therefore is a pantropical section. *Agaricus* sect. *Pantropicales*, which appeared as 'section 1' in the phylogenetic study in Chen *et al.* (2017), was proposed by Parra *et al.* (2018) and also includes species described from Asia and the Caribbean.

--- Two sections (*Minoriopsis* and *Kerrigania*) were recently proposed by Parra *et al.* (2018). In fact, these two neotropical sections already appeared in Chen *et al.* (2017) as two sister clades representing putative sections in *A.* subg. *Minoriopsis*. *Agaricus* subg. *Minoriopsis* was previously considered by Zhao *et al.* (2016) as being *Agaricus* sect. *Laeticolores* because it includes species such as *A. rufoaurantiacus* Heinem. that Heinemann (1961) placed in this section. However, the ITS sequence of the type specimen of *A. laeticulus* Callac, L.A. Parra, Linda J. Chen & Raspé (≡ *A. laeticolor* Heinem. & Gooss.-Font.), which is the type species of *A.* sect. *Laeticolores*, was recently obtained and phylogenetic analyses revealed that it does not belong to this clade but to *A.* sect. *Minores*. In addition, Chen *et al.* (2017) estimated the stem age of this clade at 31.02 Ma. Therefore, Chen *et al.* (2017) proposed *A.* subg. *Minoriopsis* and designated *A. martinicensis* Pegler (Fig. 2d) as the type species of this new neotropical subgenus.

--- The remaining section (*Cymbiformes*) was recently proposed by He *et al.* 2018a for a single species from Thailand, *A. angusticystidiatus* M.Q. He, Desjardin, K.D. Hyde & R.L. Zhao. This section is palaeotropical like its two closely related sections (*Trisulphurati* and *Crassispori*).

The 13 remaining sections include temperate species. In these sections, tropical species are unevenly distributed and often grouped into distinct subclades. According to our counts based on current available data, the proportion of tropical species is greater than 50% in *A.* sect. *Minores* and *A.* sect. *Rarolentes*, achieving around 40% in *A.* sect. *Xanthodermatei*, lower than 20% in two subgenera and three sections (*A.*

subg. *Agaricus*, *A.* subg. *Flavoagaricus*, *A.* sect. *Nigrobrunnescentes*, *A.* sect. *Spissicaules*, *A.* sect. *Subrutilescentes*), and close to zero in the five remaining sections.

The clade TRIV, that includes *A. deserticola* G. Moreno, Esqueda & Lizárraga (Zhao *et al.*, 2011) and a few species, such as *A. martineziensis* Heinem. were not included in the recent revision of the system. Therefore, the classification of these species is presently not resolved as this is also the case for a few *incertae sedis* in Zhao *et al* (2016).

Fig. 2. a. *Agaricus erectosquamosus*, LD2012165 (*A.* sect. *Flocculenti*), b. *Agaricus leucocarpus*, SCK089 (*A.* sect. *Leucocarpi*), c. *Agaricus fiardianus*, F2389 (*A.* sect. *Rubricosi*), d. *Agaricus martinicensis*, LAPAM43 (*A.* sect. *Minoriopsis*). Photo credit: b. Samantha C. Karunarathna, c. Jean-Pierre Fiard (LIP herbarium), d. Claudio Angelini.

SPECIES OF INTEREST

Agaricus sect. *Arvenses* is now ranked as *A.* subg. *Flavoagaricus*. Certain species of this subgenus such as *A. subrufescens*, *A. arvensis*, and *A. fissuratus* are cultivated and are of nutritional or medicinal interest. The three species collected in Thailand (*A. subrufescens*, *A. flocculosipes*, and *A. subtilipes* Thongklang, Linda J. Chen, Callac & K.D. Hyde) fruited on standard compost substrate (Thongklang *et al*. 2014b, 2016, Zhao *et al*. 2012b). Cultivated sporocarps of *Agaricus flocculosipes* (Fig. 3) were tasty, while *A. subtilipes* was not consumed and thus, its edibility remains unknown. The life cycle of a specimen of *A. subrufescens* was studied and hybridizations with French and Brazilian specimens were performed

(Thongklang *et al*. 2014b). Parental and hybrid strains were subsequently analyzed for their production of blazeispirol A, a spiro-triterpenoid conferring a cholesterol-lowering activity to the mycelium of *A. subrufescens*. Two other species, that are in the same 'tropical' clade as *A. flocculosipe*s and *A. subtilipes* within *A.* subg. *Flavoagaricus*, have not been formally described but are potentially interesting since their samples were collected from marketplaces in Africa by Thoen (Gui *et al.* 2015, Thongklang *et al*. 2016). Two species of *A.* sect. *Brunneopicti*, *A. bingensis* Heinem. and *A. subsaharianus* L.A. Parra, Hama & De Kesel, are also consumed by local people according to Pegler (1977) and Hama *et al*. (2010), respectively.

Fig. 3. First cultivation of *Agaricus flocculosipes*, isolate CA917, 20 November 2011 at INRA-MycSA (France) with PhD students of the Center of Excellence in Fungal Research, Mae Fah Luang University (MFLU, Thailand). a. Culture trays with, from left to right, Guinberteau J., Coldefy C., Thongklang N. (MFLU), Callac P., Chen J. (MFLU), Sysouphanthong P. (MFLU), b. and c. sporocarps.

HOW TO IDENTIFY TROPICAL *AGARICUS*?

As for any specimen of *Agaricus*, during fieldwork, it is necessary to note the odor, the discoloration of the sporocarp when rubbed or longitudinally cut, the structure and ornamentation of the annulus, and to test the Schäffer reaction on fresh tissue or as soon as possible. For tropical species, a clear and strongly positive Schäffer reaction indicates with high confidence that the specimens belong to *A.* subg. *Minores*, *A.* subg. *Minoriopsis* or *A.* subg. *Flavoagaricus*. The distinction between the tropical sections as well as diversity within these sections is based on recent data and it is still difficult to identify many species

without ITS sequence data even in the *A.* sect. *Brunneopicti* that was recently reconstructed (Chen *et al.* 2015). ITS sequences are generally sufficient to identify a species when a highly similar sequence of a specimen of reference is available in GenBank. Generally, 'highly similar' means that there is no more than one difference between the sequences. However, we do not count heteromorphisms as true differences (for example one sample has two peaks, C and T, at a given position of the chromatogram and the reference sample has only T at this position) which might reflect intraspecific allelic polymorphism. The best specimen of reference is of course a type, if possible the holotype. For most species described since 2000, the ITS sequence of their holotype is available in GenBank; those species newly described between 2000 and June 2015 are listed in Callac (2015). For species proposed before 2000, it is necessary to sequence herbarium specimens and this has already been completed for some tropical species. Species of *Agaricus* from tropical and humid subtropical regions of Asia were recently listed (Karunarathna *et al.* 2016). There are some exceptions to the rule above. More than one difference may be observed between ITS sequences of different collections within some species. Two differences are observed in some variable species or even more in cosmopolitan species such as *A. subrufescens* (Chen *et al*. 2016a, 2016b) and *A. endoxanthus* (Chen *et al*. 2016c). In contrast, two species that diverged recently from each other could share identical ITS sequences as this is the case for *A. gemellatus* Kerrigan, L.A. Parra, Cappelli & Weholt and the secotioid species *A. inapertus* Vellinga (Kerrigan 2016). Such examples indicate that morphological descriptions remain useful. In addition, a biological circumscription of the species of interest remains crucial to characterize available germplasm and develop breeding programs.

CONCLUSIONS

The number of species, sections and subgenera has recently increased and, in the new system of classification, tropical species are placed more accurately. Despite recent advances in taxonomy and phylogeny, enormous taxonomic work remains. Species diversity in Africa, South America, and Australia remains poorly known. More investigations in all these continents may allow clarification of the evolutionary history of this genus in which climate and geography seem to have been the main factors of diversification. The new system of classification should facilitate studies of *Agaricus* in the tropics and, reciprocally, such studies in various regions should allow to further improve the system of classification.

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3. AN OVERVIEW OF TROPICAL AND SUBTROPICAL SPECIES OF *Agaricus* IN MEXICO

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ABSTRACT

The genus *Agaricus* comprises around 400 species, most of which are described in temperate zones, with a smaller number found in tropical zones. Due to phenotypic plasticity of the genus, many *Agaricus* species are difficult to identify using morphological characters, so recent studies have included molecular characters for identification. While there have been some studies in tropical zones of Central America, South America and the Caribbean, there is little knowledge of the diversity of this genus in tropical and subtropical zones of Mexico. The last review of the genus for Mexico was conducted in 2011. This study analyzed species of tropical and subtropical zones in Mexico and included up to 29 species of *Agaricus* that are currently known in 12 states. The vegetation type, where most (15) species of *Agaricus* have been found, is tropical montane cloud forest. This is followed by nine species of *Agaricus* found in the deciduous and sub-evergreen tropics. Advances and perspectives in the study of the genus *Agaricus* in tropical and subtropical zones of Mexico are presented.

Keywords: cloud forest, diversity, edible fungi, tropical rain forest, trends.

INTRODUCTION

The genus *Agaricus* L. includes saprobic basidiomycetes fungi, featuring some highly valued edible species (*A. bisporus*) and others with medicinal properties (*A. subrufescens*). The genus is widely distributed and is present on all continents, except Antarctica. To date, 434 species have been described worldwide (Karunarathna *et al.* 2016). However, the most extensive studies, with keys for identification and descriptions, focus mainly on species from the temperate zones of Europe (Cappelli 1984, Parra 2008, 2013). Over the years, various studies have been published pertaining to tropical regions of America (Heinemann 1961, 1962a, 1962b, 1962c, 1993, Murrill 1918, 1942, 1945, 1946, Rick 1906, 1919, 1920, 1930, 1939, 1961), Africa (Heinemann 1956, 1961, Pegler 1966, 1968, 1969, 1977), Asia (Heinemann 1980, Pegler 1986) and Oceania (Heinemann 1982). In addition, the extensive study of Kerrigan (2016), relating to the genus *Agaricus* of North America, was published recently.

It is known that tropical regions host a wide diversity of fungi, greater than that found in temperate zones (Blackwell 2011). Furthermore, even with the few studies conducted in tropical zones, it is known that nearly half of the total number of species recorded in the genus comes from these regions (Karunarathna *et al.* 2016). This suggests that there is insufficient knowledge of the species of this genus that inhabit tropical and subtropical zones.

PHYLOGENETIC RELATIONSHIPS

One of the greatest impediments to classify these species is the recognition of sections within the genus *Agaricus*, based on morphological and macrochemical characters that have varied according to the taxonomic criteria of each author. The study of Zhao *et al.* (2011) proposed a new scheme of division into sections based on molecular characters of the genus and including species from tropical zones of Asia (Thailand), Africa and America. These authors found that up to a third of the studied species did not fit

into the eight classic sections in modern studies (Parra 2008, 2013) and proposed three new clades. Subsequently, studies in tropical regions of Asia and Oceania (Chen *et al.* 2012, 2015, Gui *et al.* 2015, Zhao *et al.* 2016) recorded new species and included phylogenetic proposals that placed the species into new tropical clades. Recently, Zhao *et al.* (2016) proposed a new classification of the genus that was divided into five subgenera and 20 sections, including some previously proposed tropical clades. As a result, infrageneric categories are gradually being clarified.

ETHNOMYCOLOGICAL IMPORTANCE IN MEXICO

In Mexico, species of the genus *Agaricus* have been used since pre-Hispanic times for food and medicine. While it is true that Herrera and Guzmán (1961) commented on the difficulty of conducting a historic analysis of edible fungi in this country, the fact that many species of fungi (including those of the genus *Agaricus*) have common or popular names and are sold in markets in some parts of the Mexican Republic. This indicates familiarity with and use of these species by consumers of mushrooms in Mexico, who go to buy them using popular names such as "hongo de Sanjuan", "Sanjuanero de llano", "llanero", "champiñón de campo", "hongo blanco", "pípila" and "mazayel", among others (Fig. 1). A study of the main edible fungi of Mexico by Herrera and Guzmán (1961) was published 55 years ago and describes seven edible species sold in various markets of Mexico City and Valle de Mexico, such as the Amecameca, and the markets of the Distrito Federal, among others. Since then, several general studies have been conducted in order to provide knowledge of this theme (Estrada-Martínez *et al.* 2009, Galván *et al.* 1998, Garibay-Orijel *et al.* 2006, Guzmán 1981, 1984, 1994, 1995, 1997, Herrera and Guzmán 1961, Pérez-Moreno *et al.* 2008, Velandia *et al.* 2008).

Figure 1. Sale of mushrooms in the market of San José in Xalapa, Veracruz.

THE GENUS *AGARICUS* IN MEXICO

The floristic composition of Mexican forests that includes species of both Neotropical and Nearctic zones, allows existence of diverse vegetation types that feature a biodiversity that is particular to each one of these forests, above all in the tropical and subtropical regions. These forests, that cover less than 30% of

the country, favor the presence of different species of fungi, especially those that present dark spores, such as the genera *Agrocybe*, *Agaricus*, *Bolbitius*, *Coprinus*, *Cortinarius*, *Galerina* and *Inocybe*, among others (Guzmán-Dávalos 2002).

While some studies are related to the genus *Agaricus*, most are focused on species that inhabit subtropical and temperate zones of Mexico (Gutierrez-Ruiz and Cifuentes 1990, Montoya-Bello *et al.* 1987, Pérez-Silva *et al.* 2011). Other studies are basically general inventories that register collections from different zones. The most complete studies are those of Guzmán (1983), who describes *A. benzodorus, A. endoxanthus, A. denisii, A. purpurellus, A. singeri* and *A. trinitatensis* of the Yucatán Peninsula. In one of the pioneering studies on tropical species of this genus, Gutiérrez-Ruiz and Cifuentes (1990) describe species of various vegetation types including those of the tropical montane cloud forest (*A. comtulus, A. essettei, A. impudicus, A. phaeolepidotus, A. moelleri, A. semotus, A. benesii, A. xanthodermus* and *A. xantholepis*), tropical low deciduous forest (*A. moelleri*), tropical sub-evergreen forest (*A. fuscofibrillosus*) and tropical deciduous forest (*A. semotus*). Finally, Medel *et al.* (2015) describe six species of tropical montane cloud forest (*A. augustus, A. comtulus, A. sylvicola, A. sylvaticus* and *A. xanthodermus*) and tropical deciduous forest (*A. phaeolepidotus*). Only *A. yucatanensis* is excluded from the list, due to the fact that the type specimen of this species deposited in the Farlow herbarium (FH, Harvard University) was reviewed by the first author of this paper who concluded that it did not correspond to a species of the genus *Agaricus*.

The last review of the genus for Mexico was conducted by Mata *et al.* (2011), who highlighted 32 species distributed across 25 states of the country. In that study, the authors mention diversity of species in tropical and subtropical zones of Mexico could be significant due to the fact that, in these regions, the genus has been insufficiently studied. Since the date of that review, only two new studies have been published (Medel *et al.* 2015, Mata *et al.* 2016) and progress in the knowledge of this genus, therefore, remains moderate.

This study includes a bibliographic review of all studies published in Mexico from 1896 to 2016. Fifty-five species have been recorded to date for temperate, tropical and subtropical forests. In this chapter, we discuss only the species known to inhabit tropical zones.

Table 1 lists 29 species of the genus *Agaricus* corresponding to records for tropical and subtropical forests. These are ordered into nine categories or vegetation types (Fig. 2). In order to group types of vegetation, equivalences were established with vegetation types according to Rzedoswki (1978). In cases where types such as tropical forest or tropical vegetation are mentioned these were conserved. Such denominations cannot have equivalents since they constitute very wide categories. The tropical montane cloud forest has the most records, with 11 species. In tropical forests (deciduous, evergreen and sub-evergreen) there are nine species and in the xerophylous scrub there are only two records. Analysis per vegetation type corroborates, at least quantitatively, the hypothesis that a non-determined number of new records will be found in that vegetation type, even including new species. This is supported by composition of the floristic kingdoms and provinces of Mexico (Rzedowski 1978) where the Neotropical kingdom coincides exactly with the location of states with the highest number of records of *Agaricus* species in tropical and subtropical zones (Fig. 3).

Table 1. *Agaricus* species recorded from tropical areas in Mexico.*

Species	States	Vegetation	Edibility	References
A. arvensis	Ver., Mor.	Tsv, Cf, Tv	(+)	8, 13, 21
A. augustus	Gro., Edo. Méx., Tamps., Ver.	Cf	(+)	3, 6, 12, 15
A. benesii	Gro.	CF	(+)	7
A. benzodorus	Q. Roo	Tv	(*)	9
A. bitorquis	*w. l.*	Tsv	(+)	8
A. campestris	Chis., Hgo., Jal., Mich., Oax., Ver.	Tsv, Ats, Cf, Thf, Tdf, Tdfp	(+)	2, 8, 10, 14, 20, 21, 22
A. comtulus	Gro.	Cf	(+)	7
A. aff. *dennisii*	Q. Roo	Tv	(*)	9
A. deserticola	Son.	Thf	(*)	17
A. endoxanthus	Q. Roo	Tv	(-)	9
A. essettei	Son.	Tdfp	(+)	18
A. fuscofibrillosus	Gro.	Tef	(+)	7
A. impudicus	Chis., Gro., Hgo., Mich., Ver.	Tef	(+)	4, 7
A. martineziensis	*w. l.*	Tsv	(+)	8
*A. moelleri (*como *A. praeclaresquamosus*)	Gro., Mor., Oax., Ver.	Cf, Tdfp	(-)	3, 7
A. osecanus	Mor.	Tsv	(+)	16
A. phaeolepidotus	Gro., Edo. Méx., Mor., Ver.	Tdf, Cf	(-)	7, 15
A. placomyces	Mich., Tamps., Ver.	Ats, Cf	(-)	1, 4, 14
A. purpurellus	Q. Roo	Tv	(+)	9
A. semotus	Gro., Mor.	Tf, Cf	(+)	7
A. singeri	Q. Roo	Tv	(+)	9
A. subrutilescens	Mich.	Cf	(+)	14
A. sylvaticus	Chis., Hgo., Jal., Mich., Mor., Oax., Tamps., Ver.	Cf, Tdf	(+)	14, 19
A. sylvicola	Hgo., Mich., Oax.	Cf	(+)	5, 21, 22
A. trinitatensis	Q. Roo	Tv	(*)	9
A. variegatus	Jal.	Tf	(+)	11
A. volvatulus	Ver.	Cf	(-)	1
A. xanthodermus	Gro., Jal., Q. Roo,Tamps., Ver.	Tdf, Cf, Tf, Tv	(-)	6, 7, 9, 15, 18
A. xantholepis	Gro., Hgo.	Cf	(*)	7

*Symbols: **States:** Chis=Chiapas, Gro=Guerrero, Hgo=Hidalgo, Jal=Jalisco, Edo. Méx=Estado de México, Mich=Michoacán, Mor=Morelos, Oax=Oaxaca, *w.l.*=without locality, Son=Sonora, Tamps=Tamaulipas, Ver=Veracruz. **Vegetation:** Thf=thorn forest, Cf=cloud forest, Tf=tropical forest, Tdf=tropical deciduous forest, Tef= tropical evergreen forest, Tdfp=tropical deciduous forest (partly), Ats=arid tropical scrub, Tv=tropical vegetation, Tsv=tropical and subtropical zone. **Edibility:** (+)=edible, (-)=toxic, (*)=unknown. **References:** 1=Chacón and Guzmán 1997, 2=Chanona-Gómez *et al.* 2007, 3=Cifuentes *et al.* 1993, 4=Cifuentes *et al.* 1990, 5=Frutis and Guzmán 1983, 6=García-Jiménez and Valenzuela 2005, 7=Gutiérrez-Ruiz and Cifuentes 1990, 8=Guzmán 1977, 9=Guzmán 1983, 10=Guzmán and García-Saucedo 1973, 11=Guzmán-Dávalos *et al.* 1983, 12=Herrera and Guzmán 1961, 13=López *et al.* 1985, 14=Mapes *et al.* 1981, 15=Medel *et al.* 2015, 15=Mora and Guzmán 1983, 16=Moreno *et al.* 2007, 17=Pérez-Silva *et al.* 2006, 18=Portugal *et al.* 1985, 19=Salvador and Guzmán-Dávalos 1988, 20=Varela and Cifuentes 1979, 21=Welden and Guzmán 1978.

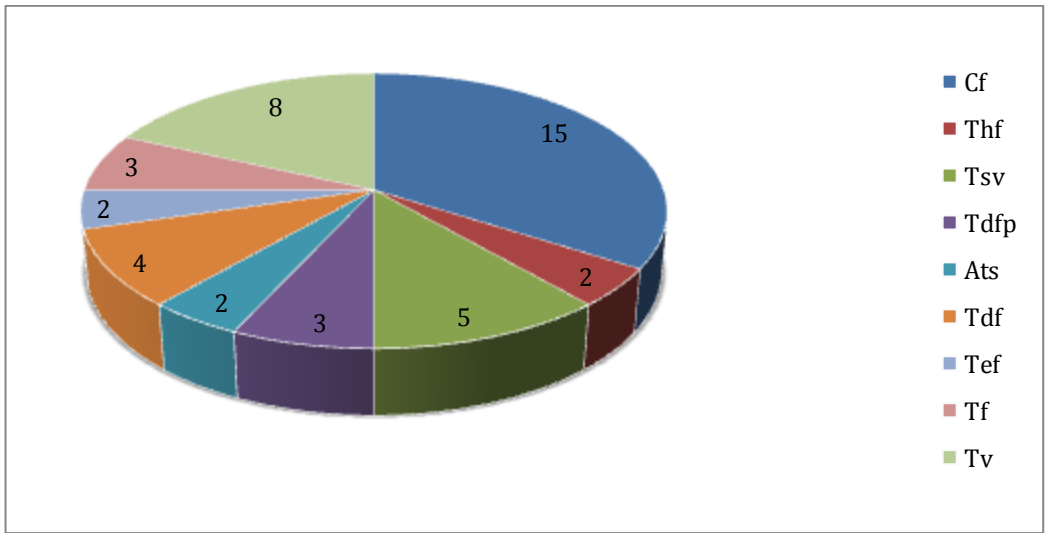

Figure 2. Records of species of *Agaricus* by vegetation type. Thf=thorn forest, Cf=cloud forest, Tf=tropical forest, Tdf=tropical deciduous forest, Tef= tropical evergreen forest, Tdfp=tropical deciduous forest (partly), Ats=arid tropical scrub, Tv=tropical vegetation, Tsv=tropical and subtropical zone.

Figure. 3. Graphical representation of the Holarctic and Neotropical kingdoms in Mexico (From Rzedowski 1978).

Geographic distributions of the species found are represented in 12 states of Mexico (Fig. 4), with Guerrero, Veracruz and Quintana Roo those that have the highest number of records (10, 10, 7 records, respectively). The fewest records are presented in Estado de México and Sonora (2 records each). This

does not mean that these states do not have diversity of species, but rather that there has been little exploration. Furthermore, there are states (e.g., Estado de México) that have more species associated with temperate forests. Of the 29 species, at least 12 have been mentioned in studies of Central and South America and that confirms some of these may be more American than European in distribution.

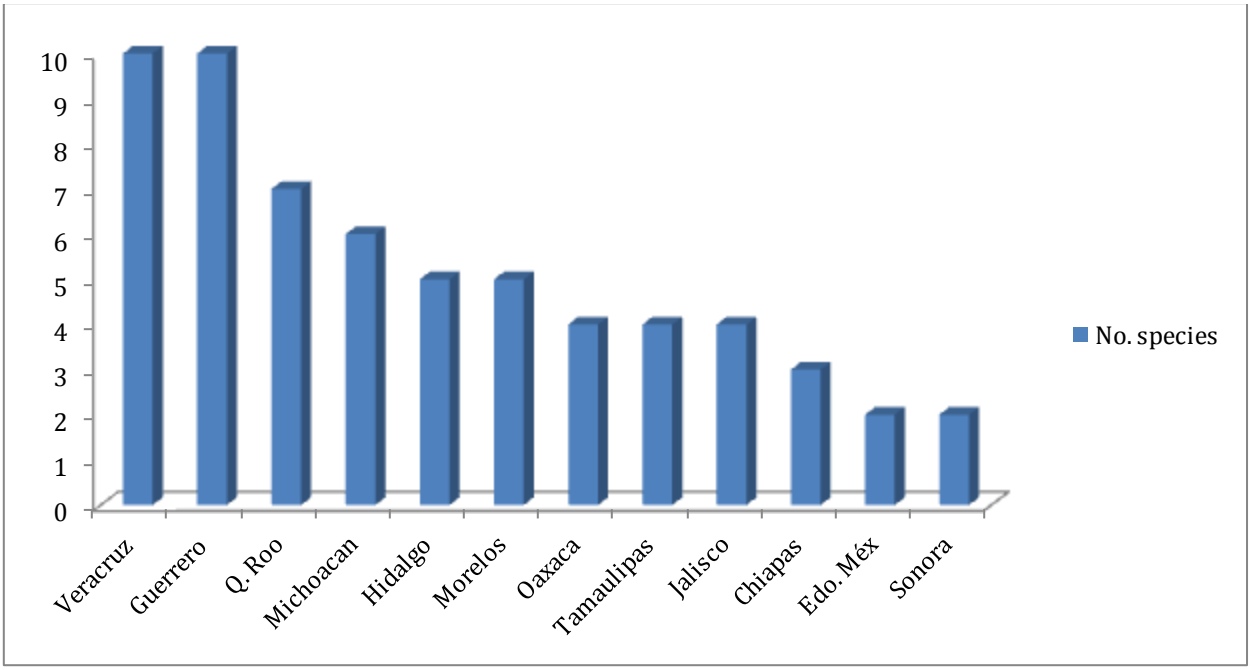

Figure 4. Records of tropical species by state.

PERSPECTIVES OF STUDIES IN TROPICAL AND SUBTROPICAL AREAS OF MEXICO

Following analysis of information obtained from the last update of knowledge of this genus in Mexico, some published studies have suggested a few guidelines for study of the genus *Agaricus* (Medel *et al.* 2015). These suggested that scarcity of studies of this genus is significant and that there is still a lack of literature to enable identification of species collected in tropical and subtropical zones. It is very important to document macro and micro morphological characters, important microchemical reactions (i.e., Schäffer and 5% KOH), odor and change of color in the context and surface of the pileus and stipe, as well as presence of structures such as rhizomorphs, in order to complete a more certain identification. Mata *et al.* (2016), in a study of wild *Agaricus bisporus* in Mexico, emphasized the need to support identifications with molecular characters, since this is a genus that is sensitive to environmental change and therefore the morphology can change within the same species to an extent that there would be variations in number of spores per basidium.

Finally, study of the genus *Agaricus* from the point of view of diversity is interesting *per se*. However, various species produce compounds of medical interest, including *Agaricus subrufescens*, the "almond mushroom" from which compounds with anti-cancer activity have been obtained (Wisitrassameewong *et al.* 2012).

Study of this genus is ongoing in Mexico and the tropical zones of that country are reservoirs of new or previously unknown species. Knowledge from these studies may lead to additional commercial

opportunities for both the agricultural and pharmaceutical industries.

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Cultivation

4. DEVELOPMENT OF THE STRAW MUSHROOM SECTOR IN CHINA

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ABSTRACT

This paper provides an historical overview of straw mushroom (*Volvariella volvacea*) cultivation, development of the straw mushroom sector and progress in research on the straw mushroom in China. Cultivation of the straw mushroom originated in southern China more than 300 years ago. Its cultivation method was introduced to Malaysia in 1930s and soon cultivation of the straw mushroom was carried out in Southeast Asia and North Africa. Cultivation methods of the straw mushroom are among the simplest of all mushroom species and its production was the third largest in the world in 1986. However, in the past three decades, development of the straw mushroom sector in China was very slow, mainly due to its low biological conversion efficiency and short shelf-life. It is anticipated that the industrialized and large-scale production of straw mushroom soon will replace the traditional cultivation methods in China in response to growing demand in the Chinese market.

Keywords: mushroom production, straw mushroom, mushroom cultivation, edible mushroom, fungal technology

INTRODUCTION

The straw mushroom [*Volvariella volvacea* (Bull.) Singer] is a species of edible mushroom belonging to kingdom fungi, division *Basidiomycota*, class *Agaricomycetes*, order *Agaricales*, family *Pluteaceae*, genus *Volvariella*. It is an important grass-rotting fungus growing on fiber wastes such as rice straw, cotton and hemp. The straw mushroom prefers high temperature and high humidity and therefore, it is traditionally an edible mushroom of the tropics and subtropics. The straw mushroom has high nutritional and medicinal values since it is rich in proteins, vitamin C and contains 18 amino acids. China is the largest producer of the straw mushroom in the world and the main producing areas of this mushroom in China are provinces in southern China and southwest China, including Guangdong, Guangxi, Sichuan, Fujian, Hunan and Jiangxi.

HISTORY OF STRAW MUSHROOM CULTIVATION

Cultivation of the straw mushroom originated in southern China, with a history of more than 300 years. As early as the Ming Dynasty (1368-1644), there was a description of straw mushroom cultivation in a book

called "Book of Planting Trees" written by Yu Zongben. Records of straw mushroom cultivation also appeared in county annals or annals of local governments in southern China in the Qing Dynasty (1644-1912), such as "Annals of Guangdong" compiled by Ruan Yuan in 1822, "Annals of Yingde County: Products" complied by Huang Peirong in 1844, "Annals of Ningde County" in Fujian Province, and "Annals of Shaozhou Prefecture" complied by Lin Shuxun in 1874. In those documents, the straw mushroom was clearly defined as a kind of wild edible mushroom that originally grew on decaying straw in southern China. Monks in the Nanhua Temple in Guangdong were the first consumers of straw mushrooms who collected them in the wild and then started to use rice straw to cultivate them in rice fields (Chang 1978, He *et al.* 2010).

According to studies by Malaysian scholar JA Baker and Thai scholar K Jalaricharana, cultivation methods of the straw mushroom were brought to Malaysia by overseas Chinese people in 1932. Soon afterwards, cultivation of straw mushrooms quickly spread to Southeast Asia and North Africa. Therefore, globally, the straw mushroom is known as the Chinese mushroom (He *et al*. 2010).

Before 1960s, a traditional method of "piling up straw and pouring manure" had been adopted in artificial cultivation of straw mushrooms. Outdoor cultivation of straw mushrooms that uses raw material piling up and cultivation had low and unstable yield, with only around 7% biological conversion efficiency. Along with development of modern biology, genetics, microbiology and environmental engineering, researchers in China, led by Professor Chang Shuting and Professor Deng Shuqun, assisted the rapid development of straw mushroom production, through continuous accumulation of technologies and research achievement. In the 1960s, a field cultivation method and a simple structure room cultivation method were developed for the first time in China by Guangdong Institute of Microbiology. The Institute introduced the concept of pure culture spawn and selected the first batch of straw mushroom strains, V20 and V23. These strains are still the main cultivars of straw mushrooms throughout the country. In the 1970s, Professor Chang Shuting of the Chinese University of Hong Kong made the first successful production of straw mushrooms using cotton waste. Along with improvement of strains and cultivation facilities, biological conversion efficiency increased to 20 to 35% and development of straw mushroom production increased rapidly. In the 1980s, bed cultivation in thermally-insulated and/or brick rooms was adopted in Guangdong, Jiangsu, Zhejiang, and Shanghai, improving the production cycle. In the late 1980s and early 1990s, bag cultivation of the straw mushroom was adopted and resulted in more efficient and stable yield. He Huanqing and his team at Guangdong Academy of Agricultural Sciences, experimented with Chinese herbal residue to cultivate straw mushrooms and this proved a success. Thus, a new source of raw material for straw mushroom cultivation became available and this method was widely adopted in Guangdong Province (He *et al.* 2010).

Compost for straw mushroom cultivation undergoes a development process from first fermentation (composting) to secondary fermentation (composting and pasteurization) and then to indoor pasteurization. When using rice straw as the main raw material and using secondary fermentation and a bed-type cultivation model, in general, the biological conversion efficiency is 10 to 20%. This is 2 to 3 times higher

than that of outdoor "piling up" cultivation. Straw mushrooms may be cropped 18 to 20 times in one year when using cotton seed hulls and/or waste cotton as the main raw materials and using pasteurization treatment method for indoor cultivation. So far, various cultivation modes of straw mushrooms have been established in various geographic regions in China. In recent years, Jiangsu Jiangnan Biotech Co., Ltd., as the representative of some enterprises in China, started the industrialized production of straw mushrooms.

ISSUES IN DEVELOPMENT OF THE STRAW MUSHROOM INDUSTRY

The cultivation method of the straw mushroom is the simplest among methods of all edible mushrooms. The straw mushroom requires the shortest time for fruiting, compared with other edible mushrooms. Raw materials for straw mushroom cultivation are abundant. Total output of straw mushrooms reached 38,000 tons in 1979 and its production scale was third highest in the world relative to button mushroom and shiitake in 1986. However, in the past 3 decades, development of the straw mushroom industry was very slow, while the industries of other mushroom species have been developing very rapidly. In 2014, China's total output of mushrooms reached 32.7 million tons, while the output of straw mushrooms was only 250,000 tons, accounting for 0.8% of total output. The main reasons for this result include biological characteristics of straw mushrooms and the influence of several factors in the process of production and distribution (He *et al.* 2010).

Strain

As the most important and most effective method for edible fungi breeding, cross-breeding has made an outstanding contribution to rapid development of China's edible mushroom industry and to helping China take the lead position in the world. However, breeders are not able to distinguish between homokaryotic and heterokaryotic mycelium or between parents and hybrids, by morphological characters, due to the fact that straw mushroom is regarded as a kind of primary homothallic fungus and its hypha has multinucleate cells and no clamp connections. Therefore, progress in straw mushroom breeding has been very slow. Very few researchers work on straw mushroom breeding and efforts so far have not resulted in improved strains (Zhao *et al.* 2015a).

Growth characteristics

The straw mushroom prefers high temperature and high humidity so it can grow well only when air temperature is above 25℃ and humidity is above 80%. In addition, its fruiting temperature is more than 35℃. These growth characteristics greatly limit large-scale cultivation and popularization.

Biological conversion efficiency

Compared with other major cultivated edible mushrooms, the straw mushroom's biological conversion

efficiency is still low. For instance, its biological conversion efficiency of natural straw substrate is less than 10%. Its biological conversion efficiency tops out at about 40%, although waste cotton, cottonseed hulls, and other materials have added to the efficiency in some cases. In comparison, the biological conversion efficiencies of oyster mushrooms *(Pleurotus* spp.), button mushroom (*Agaricus bisporus*), shiitake (*Lentinula edodes*), black ear mushroom (*Auricularia auricula*), and enoki (*Fammulina velutipes*) are all above 80% (He *et al.* 2010).

Poor storage durability

Pilei of straw mushrooms can continue to stretch and open after harvesting fruit bodies, which lower their quality and commodity value. Under conditions of conventional low temperature storage, straw mushroom fruit bodies become soft and even liquefy or decay, meaning straw mushrooms have a very short shelf life. Its poor storage durability is one of the main reasons that limit development of the straw mushroom industry.

PROGRESS IN STRAW MUSHROOM RESEARCH

Having recognized the above-mentioned factors limiting the development of the straw mushroom industry, researchers in China have been making great efforts and attempts to overcome problems limiting straw mushroom production and its industry.

Using modern molecular biology to improve breeding efficiency

Researchers at Shanghai Academy of Agricultural Sciences and Fujian Agriculture and Forestry University have completed whole-genome sequencing of the straw mushroom. Bao Dapeng (2013) conducted genome sequencing of *Volvariella volvacea* V23 by Roche 454 GS FLX and Illumina Solexa. The genome is 35.7 Mb in size and encodes 11,084 proteins based on bioinformatics prediction. Among the total predicted, 5,516 genes have been assigned either a definitive or tentative function (Bao *et al.* 2013). Comparative analysis found that there is a mating-type system in straw mushrooms that is similar to the two polar mating-type system in *Pholiota nameko*. Zou (2012) concluded that *Volvariella volvacea* is a two polar mating type mushroom after using conventional pairwise mating method and combining with morphological marker of colony and SV molecular marker to verify the paring result. Xue Chengqin *et al.* established specific molecular markers on the basis of straw mushroom A factor, and thus, established molecular marker-assisted breeding technology system of straw mushroom (Xue *et al.* 2013). Compared with the conventional cross-breeding method of straw mushroom, molecular marker-assisted breeding technology system can effectively improve the probability of mating success and obtain fertile stains, because many hybrids do not produce fruit bodies in conventional cross-breeding. Therefore, application of molecular marker-assisted breeding technology system can greatly improve the efficiency of cross-breeding (Xiong *et al.* 2014, Zhao *et al.* 2015).

Studies on molecular biology of the straw mushroom are mainly focused on application of a variety of molecular markers in strain identification. Through identification of gene library of straw mushroom, Chen Mingjie *et al.* (1996) found that the straw mushroom genome has a high percentage of repetitive sequences. Among these repetitive sequences, the moderately repetitive sequences are unique in different interspecific and intraspecific strains, so they can be used to construct molecular markers (Chen *et al*. 1996). The highly repetitive sequences may be more conservative DNA sequences that have important values in the study of evolution and classification of fungi. Fu Junsheng *et al.* (2010) screened hereditary differences in bands of single-spore strain of parents by SRAP, then, converted hereditary difference bands to SCAR markers to identify hybrids (Fu *et al*. 2010). Chen *et al.* (2011) identified straw mushroom hybrids using RAPD technique that made use of the phenomenon that parents' specific bands are always present in their offspring.

Use of modern molecular biology to improve biological conversion efficiency of the straw mushroom

Straw mushrooms can produce a variety of enzymes that degrade cellulose to glucose, including β-1, 4-endoglucanase, β-glycosidase, and cellobiohydrolase. In recent years, study of the isolation, cloning, and gene transfer of a cellulase gene has become a hot topic of research on molecular biology of straw mushrooms in the world. Based on accomplishment of whole-genome sequencing project of *Volvariella volvacea* and establishment of the genome-wide framework map of *Volvariella volvacea*, Wang Menglan *et al.* (2014) found the genes encoding hemicellulase degrading enzymes from whole-genome by bioinformatics analysis. By comparing structures of hemicellulase degrading enzymes of straw mushroom of some grass-rotting fungi and wood-rotting fungi and by analyzing the level of gene expression and enzyme activity, researchers attempted to identify differential genes between straw mushroom and other edible mushrooms and to explore reasons for low biological conversion efficiency of straw mushroom. It is speculated that xylanase may be a crucial enzyme in straw mushroom hemicellulase, and xynII may be an important gene in the process of degrading hemicellulose (Wang *et al*. 2014, Zhao *et al*. 2015)

Bao *et al.* (2013) found 11 laccase isozyme genes (lac genes) and 4 manganese peroxidase genes in straw mushrooms, but did not find lignin peroxidase genes. Lignin peroxidase genes, that are important for lignin degradation, are absent in straw mushroom genome so this may be associated with straw mushroom's poor ability to utilize lignin. Reports associated with ligninolytic enzymes in straw mushroom are relatively few and mainly focused on laccase that has the largest amount in straw mushroom, especially on laccase gene cloning and expression (Wu *et al*. 2014). In the study by Zhu (2013), 11 laccase isozymes genes were found in straw mushroom genome and laccase gene expression was detected at the pinhead, button, egg, elongation and mature stage of straw mushroom. The results show that laccase gene transcription can occur at all stages, but expression levels at different stages are dissimilar. It is speculated that those lac genes may play a key role in morphogenesis of straw mushroom fruit bodies (Zhu *et al*. 2013).

Improving product quality and freshness-keeping technology of the straw mushroom

Initially, many studies were focused on low temperature storage of the straw mushroom while a number of studies were directed toward cultivation management and strain domestication. For instance, plastic tunnels, arch sheds, ground sheds and other protected cultivation measures are used to conduct spore domestication at low temperature and induced domestication by cultivation in spring season. But these measures can only play a mitigation role when temperatures change and the low temperature tolerance of straw mushroom strains is not stable. Shelf life of straw mushrooms is still a problem, although some measures such as 60 Co-γ-irradiation, method of charging air and exhausting oxygen and modified atmosphere storage can extend the shelf life of straw mushroom to 74 hours (Lu *et al*. 2013).

Rapid development of modern molecular biology and its applications in various domains of biology has brought new approaches and ideas for theoretical studies on straw mushrooms and for genetic improvement of strains. Chinese researchers also conducted studies on straw mushroom autolysis at low temperature. However, the fundamental cause and role of autolysis at low temperature remains unknown. By focusing on protease, proteins and nucleic acids, through comparative analysis of mRNA differential display technique and protein spectrum (Chen *et al*. 2001), Chen *et al*. (1998) screened and cloned a low temperature response gene. Then they analyzed the change of gene expression at low temperatures, conducted expression validation after obtaining low temperature mutant straw mushroom strain and preliminarily established a low temperature response model on straw mushroom V23 strain. Low temperature can damage cell membranes of V23 strain resulting in autolysis leading to death. Synthesis of lipids and other components on the plasma membrane is accelerated in a low temperature mutant strain, thus improving cell membrane fluidity and allowing the mutant stain to survive longer at low temperature (Sun *et al.* 2005, Sun *et al.* 2012, Jiang *et al.* 2014). Guo *et al*. (2005) cloned and isolated freezing stress induced expression gene of straw mushroom by cDNA-AFLP technology and conducted sequencing and structure analysis of DNA sequence.

With the development of genetic engineering, it was found that the time of low temperature storage of straw mushroom can be effectively extended by transgenic manipulation. Guo *et al.* (2005) used a gene gun to transfer an antifreeze protein gene into straw mushroom and obtained transgenic straw mushroom by hygromycin resistance selection. Results of a low temperature stress experiment show that the transgenic mushrooms have a strong tolerance to low temperature and this tolerance is stable over generations (Guo *et al.* 2005*)*. Based on these experiments and findings, using antifreeze protein gene *afp* as target gene and selectable marker gene for straw mushroom transformation, Wang *et al.* (2010) screened transformants at 0℃ and obtained cold resistant transgenic straw mushrooms without selectable markers. Therefore, transgenic manipulation is one of the ideal approaches to develop new strains of straw mushroom that are suitable for low temperature storage (Wang *et al.* 2010).

DEVELOPMENT PROSPECT OF INDUSTRIALIZED CULTIVATION OF STRAW MUSHROOMS

With economic development and improvement of living standards in China, consumers are demanding fresh, healthy and safe agricultural products. Thus, market demand for edible mushrooms is also changing. In some rural areas, the edible mushroom industry is playing an important role in helping farmers alleviate poverty and generate more income. The development of the edible mushroom industry is supported by the Chinese Central Government and local governments in a bid to build new rural areas in China and realize the sustainable development of agriculture and the rural economy.

The straw mushroom is native to regions with high temperature and high humidity in southern China and it needs very strict conditions of temperature and humidity for storage or its nutrients will be lost quickly. Therefore, if transporting straw mushrooms from southern China to northern China, the nutritional value of straw mushrooms will decrease and costs will be increased. The supply of fresh straw mushrooms is limited to certain regions, mostly in southern and southwest China, while the demand for fresh straw mushrooms in northern China is very strong and the market potential is very large. In order to satisfy new market demand and enhance market competitiveness of products, upgrading current production modes and adopting industrialized, large-scale production is the direction of development of the straw mushroom industry in China. In recent years, some Chinese enterprises have already started industrialized production of straw mushrooms. It is expected that industrialized and large-scale production of straw mushrooms will replace traditional cultivation methods in China, in efforts to satisfy growing demand in the Chinese market.

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5 ENHANCED PRODUCTION OF THE MEDICINAL MUSHROOM *Agaricus subrufescens* PECK

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ABSTRACT

Agaricus subrufescens Peck [*A. blazei* (Murrill) ss. Heinemann] has been studied by researchers in several countries (Brazil, USA, China, Taiwan, etc.) primarily because of its medicinal and pharmacological properties. Cell walls of the fungus contain polysaccharides called β-glucans that have structural and other functions. Several studies highlight the importance of *A. subrufescens* medicinal properties. It was traditionally used to treat many common diseases like atherosclerosis, hepatitis, hyperlipidemia, diabetes, dermatitis and cancer. Beneficial properties of *A. subrufescens* include tumor growth reduction, immune modulatory activities, immune stimulatory effects, antimicrobial and antiviral activities and anti-allergy effects. In this work, various aspects of production of *A. subrufescens* are presented. Production of this edible medicinal mushroom has generated a notable interest worldwide in the last few years, becoming increasingly popular and as a result has expanded into other countries (Spain, France, South Korea, etc.). This is mainly due to its high international market price that is related not only to the remarkable medicinal properties contributed by high content of bioactive compounds but also to the culinary value added by its slightly nutty pleasant aroma.

Keywords: *Agaricus blazei*, medicinal mushrooms, production, compost, casing

INTRODUCTION

In recent years, production of the mushroom *Agaricus subrufescens* Peck has aroused increasing interest around the world, achieving great popularity. Its medicinal and culinary properties make it possible to foresee a rapid expansion of production around the world.

It is frequently referred to in the literature as *Agaricus blazei* (Murrill) ss. Heinemann or *Agaricus brasiliensis* Wasser although these names are presented, not without some controversy, as incorrectly applied or illegitimate (Kerrigan 2005, Wisitrassameewong *et al*. 2012). In Brazil, *A. subrufescens* is commonly known as "Cogumelo do Sol®", "Cogumelo Medicinal", "Cogumelo de Piedade" "Cogumelo de Deus", "Portobello de almendra" or simply as "Blazei", while in the USA, it is known as the "Almond mushroom" and as "Royal Sun Agaricus", in Japan as "Himematsutake", "Agarikusutake" and "Kawarihiratake", in Spain as "Champiñón del Sol" and in China as "Ji Rong Song" (Kopytowski Filho *et al*. 2006, Firenzuoli *et al*. 2007; Moukha *et al*. 2011).

The tradition and history of this mushroom in Brazil strongly represent an important mycological relationship between growers and merchandizers. "Blazei" is associated with mushrooms harvested in the city of Piedade (Fig. 1) in the 1960s and was originally collected by Mr. Furumoto. A detailed description of the history of *A. blazei* in Brazil can be found in the chapter published by Zied *et al*. (2012).

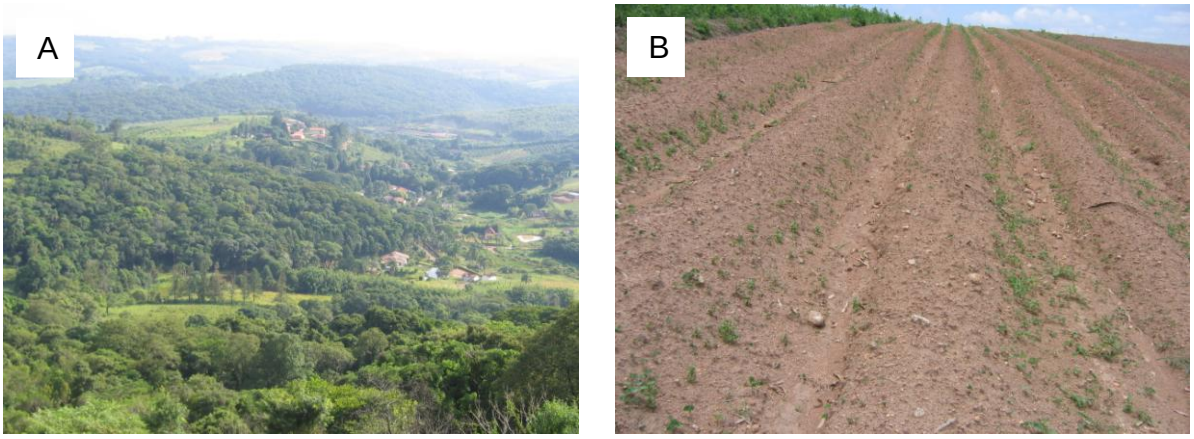

Figure 1. A) City of Piedade, region of Mata Atlantida (it is possible to visualize some specific growing houses for the production of *A. subrufescens*), B) First field crops exposed to environmental conditions that possibly gave the popular name of Sun Mushroom (substrate covered with soil ready for production).

Currently, only a few countries produce *A. subrufescens* on a commercial scale. Its production is now well-established in Brazil, Japan, China and Korea (Gregori *et al*. 2008). It has generated a notable interest worldwide in the last few years becoming increasingly popular and as a result expanding into many countries. This is mainly due to its high international market price in comparison to other mushrooms that is related not only to the remarkable medicinal properties contributed by high content of bioactive compounds but also to the culinary value added by its slightly nutty, pleasant aroma (Largeteau *et al*. 2011).

Besides fresh fruit bodies, this mushroom is mainly processed in a dried and powdered form, in capsules or pills or as an extract. Furthermore, it has been used as additive ingredients in cosmetic products (Stamets 2000, Wisitrassameewong *et al*. 2012).

Several studies recently reviewed by Wisitrassameewong *et al*. (2012) highlight the importance of *A. subrufescens* medicinal properties. It was traditionally used to treat many common diseases like atherosclerosis, hepatitis, hyperlipidemia, diabetes, dermatitis and cancer (Firenzouli *et al*. 2008). Among the beneficial properties from *A. subrufescens* that have been reported are tumor growth reduction, immune modulatory activities, immune stimulatory effects, antimicrobial and antiviral activities and anti-allergy effects (Wisitrassameewong *et al*. 2012).

In fact, in Brazil a methodology was never established regarding cultivation practices to consistently produce relatively high levels of β-glucans in the basidiomas until the publication of Zied *et al.* (2014). The authors clearly detailed the influence of strain, compost formulation, type of casing layer and environment of production on the total amount of β-glucans found in the dehydrated basidiomas.

Zied *et al*. (2014) characterized all stages of production according to the amounts of β-glucans in the basidiomas and the biotechnological behavior in its production. They concluded that the major contributors to differences in β-glucan content were as follows (in descending order): 1) strain (35.8%), 2) casing layer (34.5%), 3) environment of production (15.7%) and 4) type of compost (9.9%). On the other hand, variations in yield were mainly affected by the environment of production (82.1%), followed by strain (81.3%), casing layer (49.1%) and type of compost (15.2%). Camelini *et al*. (2005) also conducted research that showed the amount and structural characterization of β-glucans in basidiomas varied with different maturation stages. The amount of (1,3)-β-glucans in the mature stage was higher than in the immature stage.

PRODUCTION PROCESS

The processes and techniques previously established for the production of *Agaricus bisporus* (Lange) Imbach have been adopted for *A. subrufescens* cultivation (Martos *et al.* 2017). Parameters, such as the conditions of growing in the different stages of the cycle (temperature, relative humidity, carbon dioxide concentration, light exposure), materials, composting processes, moisture content and different aspects of casing layers and fruit-formation should be examined to increase yields and adapt the crop to specific conditions native to various countries.

In order to improve production of this crop and to provide guidance to growers, the following considerations and practical aspects should be considered for commercial production.

SPAWN

Strains used for production of *A. subrufescens* in Brazil are marketed as varieties collected indigenously that were selected through anthropization and adaptation to the cultivation conditions of the growers (type and formulation of the compost and local environmental conditions). The consequences are substantial variability in yield, a long growing cycle and a lack of control over specific growth characteristics of the strains.

Production of spawn on grain (wheat or rye) is carried out similarly to *A. bisporus*. Figure 2 outlines the necessary steps for production of inoculum i.e, sub-culturing and production of mother spawn in Petri dishes using culture medium and production of grain spawn and spawn in bottles and high-density polyethylene plastic bags using cereal grains.

COMPOST

Production of compost is carried out in two phases, similar to the method developed for *A. bisporus*, although the compost obtained should have a lower total nitrogen content (1.15-1.45%), providing a higher C/N ratio (25-27:1 final of Phase II). In Brazil, the traditional process of composting has been widely practiced by growers (Fig. 3) following the steps of: pre-wetting (4-7 days), fermentation (formation of the windrow 2 m wide x 2 m high, with intervals of turning every 2-3 days), pasteurization (58±2°C) and physical, chemical and biological conditioning (47±2°C) (Eira, 2003). Although the bibliographic references report a wide range of materials, characteristics for the growth substrates and C:N values higher than those commonly used for the production of *A. bisporus* are recommended. Several strains evaluated have shown good adaptation to production on different composts commercialized for mushroom growing by several composting plants in Spain (Pardo-Giménez *et al*. 2014a).

As for compost load densities, a range between 60 and 70 kg m^{-2} may be considered suitable.

Application of commercial supplements to compost at the time of spawning may provide increases in biological efficiency.

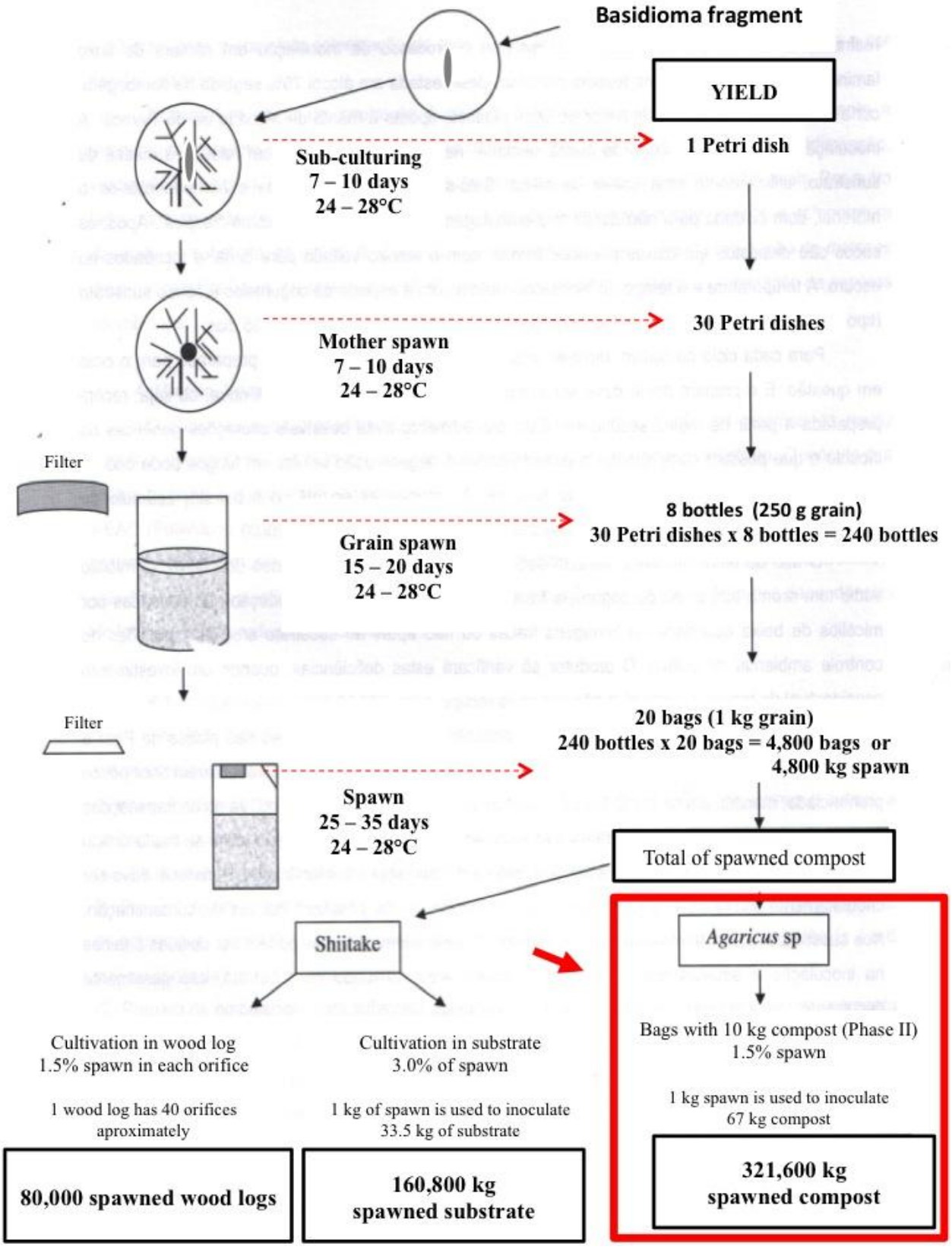
Basidioma fragment
YIELD
1 Petri dish
Sub-culturing
7 – 10 days
24 – 28°C
30 Petri dishes
Mother spawn
7 – 10 days
24 – 28°C
Filter
8 bottles (250 g grain)
30 Petri dishes x 8 bottles = 240 bottles
Grain spawn
15 – 20 days
24 – 28°C
Filter
20 bags (1 kg grain)
240 bottles x 20 bags = 4,800 bags or
4,800 kg spawn
Spawn
25 – 35 days
24 – 28°C
Total of spawned compost
Shiitake
Agaricus sp
Cultivation in wood log
1.5% spawn in each orifice
1 wood log has 40 orifices
aproximately
Cultivation in substrate
3.0% of spawn
1 kg of spawn is used to inoculate
33.5 kg of substrate
Bags with 10 kg compost (Phase II)
1.5% spawn
1 kg spawn is used to inoculate
67 kg compost
80,000 spawned wood logs
160,800 kg
spawned substrate
321,600 kg
spawned compost

Figure 2. Sequence of procedures used for preparation of matrix and spawn and potential yield from a single basidioma fragment, where in approximately 70 days a quantity of mycelium capable of inoculating 321,600 kg substrate is produced.

Figure 3. A: Phase I composting, wood structure used to make the windrows (2 m wide by 2 m high), bales of *Brachiaria* sp. and perforated grid on the floor where compost is heaped above for air circulation. B: General view of the pasteurization tunnel (Phase II), a wooden platform where air circulates from the bottom up through the compost. It is also possible to see in the photo the air outlet, which aims to eliminate excess ammonia and reduce the temperature at time of spawning.

PRODUCTION OF MUSHROOMS

Production of the Sun mushroom in Brazil is carried out by farmers at the family level and by mid-level mushroom growers. The number of mushrooms produced monthly at the family level may reach an average of up to 60 kg of dry mushrooms. Above this value, professionalized production has been defined with sectors of composting, production and mushroom processing areas, where the average production is around 150 kg of dried mushrooms per month.

Usually, facilities used in the production process have a low technological level, do not have adequate thermal insulation and do not usually have automated equipment. Thus, it is difficult to induce programmed fruit-formation.

Producers have been investing recently in facilities with a more controlled environment (Fig. 4) to allow production during all times of the year. Production in open fields (Fig. 1B) has not been carried out in Brazil since the beginning of the 2000s.

Cultivation stages are the same as those for *Agaricus bisporus*: Spawning and filling, spawn run, casing, pinning, fruit-maturation and harvesting, emptying, cleaning and disinfection. The remarkable feature is that the environmental conditions in each of these stages for *A. subrufescens* differ substantially from those applied for *A. bisporus*. During cultivation, optimum temperature for mycelial growth is around

28±2 °C, while for development of basidiomata values between 24 and 27°C are recommended. Also, high relative humidity and a high level of fresh air are required in the growing room. The growing cycle lasts an average of 120 days (period considered after addition of the casing layer) that allows the producer to perform three annual cycles per growing room.

Figure 4. A: Modern production buildings with environmental control in the mountainous region of Mata Atlantica. B: Internal view of a growing room that has thermal insulation and wooden benches containing 3 levels. Each room is 25 m long, 7 m wide and 3 m high.

CASING

Figure 5. Production using casing soils of various textures. A: Mixture of loam soil with silt clay soil, 3:1V/V. B: Silt clay soil only.

Application of a casing layer on compost colonized with mycelium is an essential operation in commercial production of both *A. bisporus* and *A. subrufescens*. This layer is where the change from vegetative to reproductive growth occurs. In the case of *A. bisporus*, numerous materials have been used for this purpose, with different types of peat moss being the most widely used in the world, mainly due to its exceptional structural and water retention properties (Yeo and Hayes, 1979). In the case of *A. subrufescens*, materials used are based, in most cases, by availability in producing countries. Casings are

usually based on mineral soils (different textures, Fig. 5) and different types of local peat, although we can find other materials such as charcoal, sawdust, sand, vermiculite, pine bark and coconut fiber pith, among others (Silva *et al*. 2007, Siqueira *et al*. 2009, Cavalcante *et al*. 2008, Colauto *et al*. 2010, Zied *et al*. 2010, Zied 2011, Zied *et al*. 2011).

Our experience indicates that peat-based casings (Fig. 6) are preferable to those based on mineral soil (Pardo-Giménez *et al*. 2014b). This is justified if we consider that, in the course of the growing cycle, there is a high degree of evaporation associated with high ambient temperature, high rate of ventilation required (CO_2 concentrations between 650 and 700 ppm) and long cycle duration (4 to 5 flushes are harvested at 12 to 14-day intervals). The ruffling operation, performed 5 days after application of casing on compost, provides a significant increase in total yield.

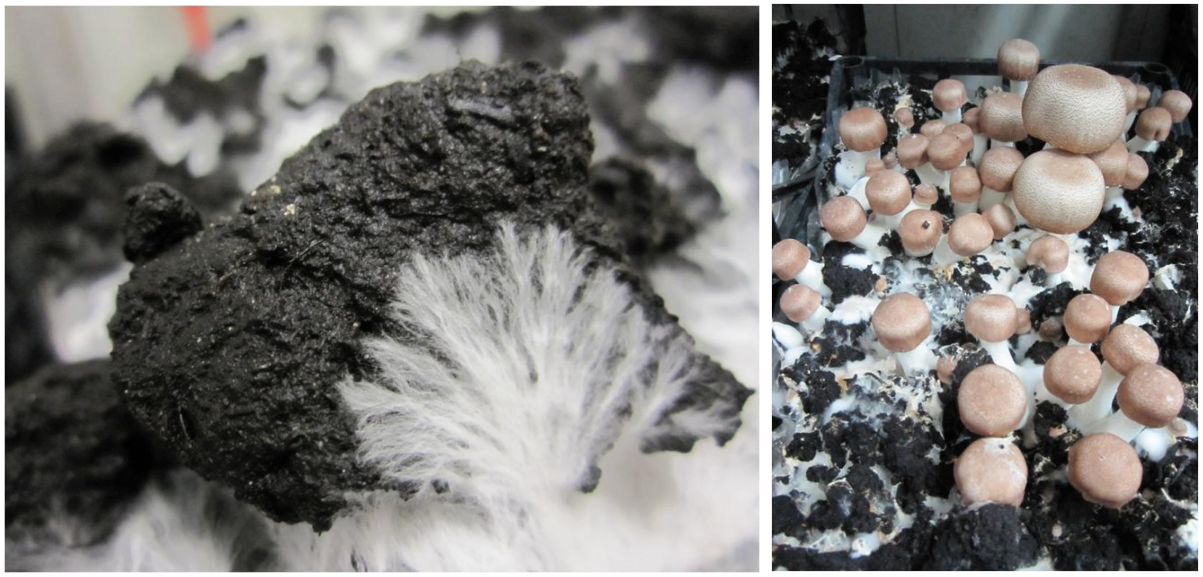

Figure 6. Use of Dutch Commercial Casing in production of *A. subrufescens*. This casing has a greater water holding capacity, high porosity and it provides a reduction in the irrigation program when compared to soil-based casing.

BASIDIOME FORMATION

Regarding induction of fruit formation, two different conditions can be initially proposed, applicable for successive flushes of mushrooms. A rapid induction that we might call "aggressive", adapted from Kopytowski Filho and Minhoni (2007) and a slow one, adapted from Kopytowski Filho *et al*. (2008), is less demanding *a priori* regarding the cooling capacity of the air conditioning system (Fig. 7 and 8). A third possibility, later evaluated in order to reduce the energy cost associated with the significant decrease in temperature required for induction, is what we termed moderately slow induction, reducing the amplitude of the applied temperature range (Fig. 9).

Although the results, in terms of biological efficiency, do not differ significantly between different induction conditions, the production pattern in terms of temporal distribution of the same is substantially modified. Thus, rapid induction allows greater control over the crop by concentrating production of each flush in fewer days. Intervals of flushes are between 12 and 14 days in this case. Slow inductions, on the other hand, provide a greater number of harvest days that may be beneficial in some cases in terms of manpower needs.

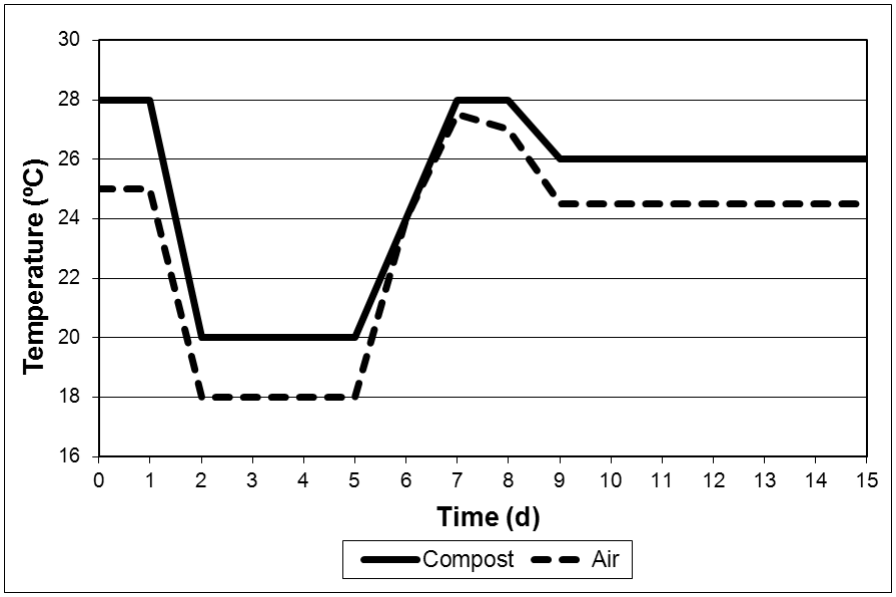

Figure 7. Control of internal and external temperatures of compost during induction of fruit-body formation by fast cooling. Reduction of compost temperature from 28 to 20°C is accomplished in one day and is maintained for 3 days at 20°C.

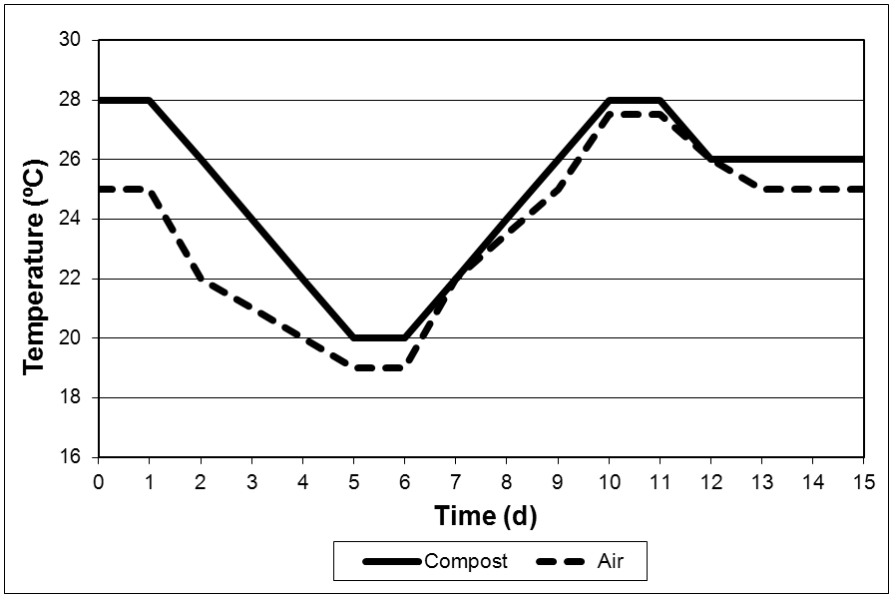

Figure 8. Control of internal and external temperatures of compost during induction of fruit formation by slow cooling. Reduction of compost temperature from 28 to 20°C occurs over four days and then is held for 1 day at 20°C.

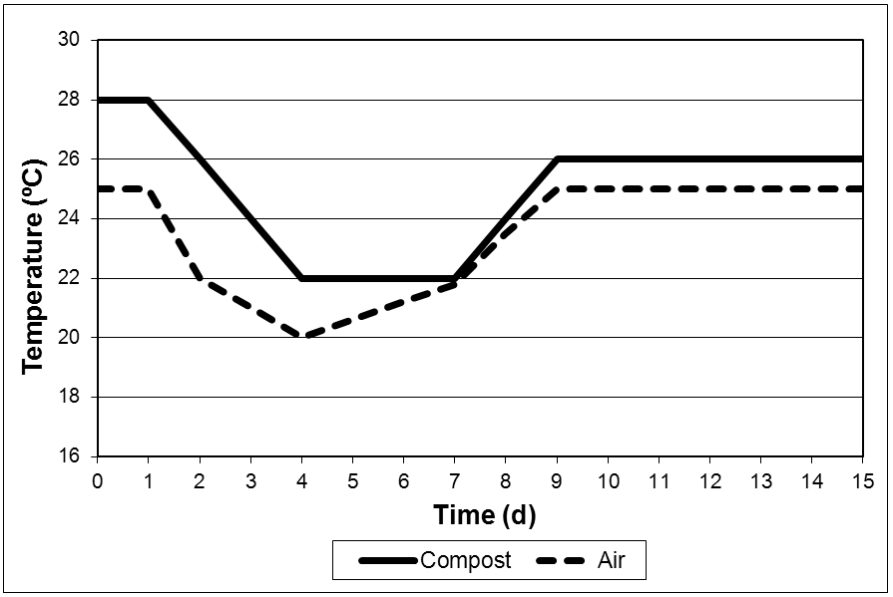

Figure 9. Control of internal and external temperatures of compost during induction of fruit formation by moderate cooling. Reduction of compost temperature from 28 to 22°C occurs over three days and then is held for 3 days at 22°C.

YIELD OF MUSHROOMS

Biological efficiency reported in the consulted literature is in a range of 13.1 to 60.4 kg dt^{-1}, with an average value around 35 kg dt^{-1}. According to Silva *et al.* (2007), in Brazil it is considered that productivity, expressed as dry matter of mushrooms with respect to the fresh weight of compost, must be at least 1% for the crop to be economically viable. Translated to biological efficiency, it should exceed the threshold of 25 kg dt^{-1} compost. Our experience shows that, under climatically controlled conditions, and depending on different factors associated with the production process (compost and casing type, schedule of operations, watering program, induction of fruit formation conditions, etc.), biological efficiency values of 50 to 70 kg dt^{-1} may be obtained.

After harvest, excess soil must be removed from the base of the stipe, then basidiomata are washed, cut and dehydrated (Fig. 10). *A. blazei* mushrooms are usually commercialized dehydrated, whole or crushed (flour, tablets, capsules, tea bags, etc.) and also combined with other foods (coffee, chocolate, honey, etc.). It should be noted that there are standards of commercialization for the export of mushrooms. Detailed information on the subject may be found in Zied *et al.* (2017).

Figure 10. A: Freshly harvested mushrooms with residue of the casing layer and fragments of mycelium at the base of the stipe. B: Washed mushrooms cut in half showing brown color absent. C: Mushrooms in trays that are placed in a dehydrator. D: Mushroom dehydration room; each dehydrator (covered by aluminium door) has an electric resistance heating system and forced ventilation and has a capacity of 100 kg of mushrooms per day. E: Dehydrated mushrooms sliced in half (light color and without signs of oxidation, standard for export) and powder and capsules, ready to be marketed.

FINAL CONSIDERATIONS

Requirements regarding necessary growing conditions and particularly, high ambient temperature, make production of *A. subrufescens* an alternative for consideration by growers in summer months. Limited technological capacity of some cultivation facilities along with high energy costs, limits production significantly in summer season. These same facilities could be used by producers to carry out a crop cycle of *A. subrufescens* in those months. Production technology and substrate processing and crop cycle management for *A. bisporus* is mostly applied to *A. subrufescens*, so growers should have few problems switching to this crop. In addition, conditions of commercialization of this mushroom, mainly dehydrated, avoid the limitation that supposes a short shelf life that generally is encountered with fresh mushrooms. This allows staggered commercialization throughout the year.

ACKNOWLEDGMENTS

We would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP-2015/15306 and 2015/24788-1) in Brazil and the Diputación Provincial de Cuenca and the Consejería de Agricultura, Medio Ambiente y Desarrollo Rural of Castilla-La Mancha in Spain.

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6. ARTIFICIAL CULTIVATION OF THE MEDICINAL MUSHROOM *Sparassis latifolia*

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ABSTRACT

Sparassis latifolia is an edible and medicinal mushroom that is cultivated in East Asia. It contains important bioactive molecules that have several biological effects including antiangiogenic activity, tumor-suppressing effect, etc. This review presents a comprehensive summary of the genomics, proteomics, biological characteristics, nutritional physiology, breeding and artificial cultivation of *S. latifolia*.

Keywords: *Sparassis latifolia*, genomics and proteomics, intrinsic and extrinsic factors, industrial cultivation

INTRODUCTION

Sparassis latifolia (also known as hanabiratake in Japanese or cauliflower mushroom in English) is an edible and medicinal mushroom that is cultivated in Japan, Korea and China. Taxonomy of *S. latifolia* is as follows: Fungi, Basidiomycota, Agaricomycetes, Polyporales, Sparassidacea, *Sparassis* (Li *et al.* 2010). The color of *S. latifolia* can range from white to creamy white and its unique shape and appearance can be described as similar to a large petal or a head of cauliflower, from which it has been given its popular name (Figure 1). *S. latifolia* is a brown-rot fungus that grows on stumps of coniferous trees (pine or larch) during the summer and autumn seasons and is widely distributed throughout the Northern Temperate Zone (Kimura 2013, Kim *et al.* 2013). It is a rare species of edible and medicinal mushroom and produces highly nutritious basidiocarps that are more precious than *Cordyceps sinensis*, *Morchella esculenta* and *Truffle*.

S. latifolia contains a remarkably high concentration of *β*-glucan as measured by an enzyme method of the Japan Food Research Laboratories (Ohno *et al.* 2000). *β*-glucan content from stipes and petals of *S. latifolia* is over 40% with its content of stipe significantly higher than that of the petal (Lian *et al*. 2014). In addition, *β*-glucan from the basidiocarp of *S. latifolia* has many biological and pharmacologic activities including antiangiogenic activity (Harada and Ohno 2008, Yamamoto *et al.* 2009), tumor-suppressing effect (Ohno *et al.* 2000, Hasegawa *et al.* 2004), etc. Polysaccharide fractions have been prepared from cultivated *S. latifolia* and its major structural units and biological activities of the extracts have also been examined (Tada *et al.* 2007).

Figure 1. Fruit body of *Sparassis latifolia*

Because of the nutritional and medicinal value given above, *S. latifolia* has attracted interest of researchers throughout the world and, as a result, consumer demand has increased. However, the production of *S. latifolia* is still very low and industrial cultivation techniques of *S. latifolia* have been limited to a few commercial farms because of its slow mycelial growth into cultivation substrate. In addition, control of environmental conditions is also required for primordium formation and growth of fruit bodies, which is hardly achievable on general commercial farms (Kim *et al.* 2013). Up to now, there are only three farms that have artificially cultivated this mushroom in China so the total fresh production of *S. latifolia* is just over 2.5 tons/d (Figure 2). Our previous study has shown that *S. latifolia* requires about 120 days to complete its crop cycle from inoculation to harvest, which is longer than *Flammulina velutipes*, *Hypsizigus marmoreus* and *Pleurotus eryngii* cultivation (Lin *et al.* 2012). *S. latifolia* yield is a complex trait and the mushroom's survival and multiplication are related to a number of factors including intrinsic and extrinsic factors and their interactive effects. Intrinsic factors include carbon and nitrogen sources, minerals, pH, type of mushroom spawn, substrate composition, moisture, ratio of carbon to nitrogen, particle size, etc. Extrinsic factors include temperature, humidity, luminosity and air composition of the surrounding substrate, such as concentration of oxygen and carbon dioxide.

Considering nutritional characteristics and growth conditions, domestication of *S. latifolia* has been a difficult endeavor for a long time. The Institute of Edible Fungi, Fujian Academy of Agricultural Sciences (FAAS) has focused on *S. latifolia* breeding, biological characteristics, nutritional physiology, submerged fermentation and commercial cultivation aspects since 2004. In this review, we provide a comprehensive insight into the above-mentioned factors of *S. latifolia* with the intent to provide knowledge to people for improving yield and quality of *S. latifolia*.

Figure 2. Industrial cultivation of *S. latifolia* in China

GENOMICS AND PROTEOMICS OF *S. latifolia*

Genome features

We sequenced the whole genome of *S. latifolia* and further assembled it into a 48.13 Mb draft genome. The *S. latifolia* genome is similar in size to the genomes of several other species from the order Polyporales, including *Trametes versicolor* (44.79Mb), *Wolfiporia cocos* (50.48 Mb) (Floudas *et al.* 2012), *Phanerochaete carnosa* (46.29 Mb) (Suzuki *et al.* 2012) and *Polyporus brumalis* (45.72Mb) (http://genome.jgi.doe.gov/Polbr1/Polbr1.info.html). We further mapped the genome to Eukaryotic Clusters of Orthologs (KOG), Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to further characterize the predicted proteins.

Proteomes

We investigated protein expression at different developmental stages of *S. latifolia* using Isobaric Tags for Relative and Absolute Quantification (iTRAQ)-coupled 2D LC-MS/MS (Two-Dimensional Liquid Chromatography Tandem Mass Spectrometry). A total of 2305 reliable proteins were identified using Q-Exactive mass spectrometry and ProteinPilot search engines and 2219 of these proteins had a quantitative dimension. Of quantitatively different proteins, 104 were significantly up-regulated and 142 down-regulated at the early fruiting stage (80 days following inoculation) and 155 were significantly up-regulated and 460 down-regulated at the fruiting stage (115 days following inoculation), compared with the primordium stage (60 days following inoculation), respectively. Data from gene ontology (GO) molecular functional analysis revealed that differentially expressed proteins were mainly involved in catalytic activity, protein binding and hydrolase activity (Figure 3). Significantly enriched KEGG pathways included those related to carbon metabolism, biosynthesis of amino acids, ribosome pathways

and glycolysis/gluconeogenesis (Figure 4). Furthermore, the numbers of differentially expressed proteins related to signal transmission and transcription factors were 27 and 7, respectively.

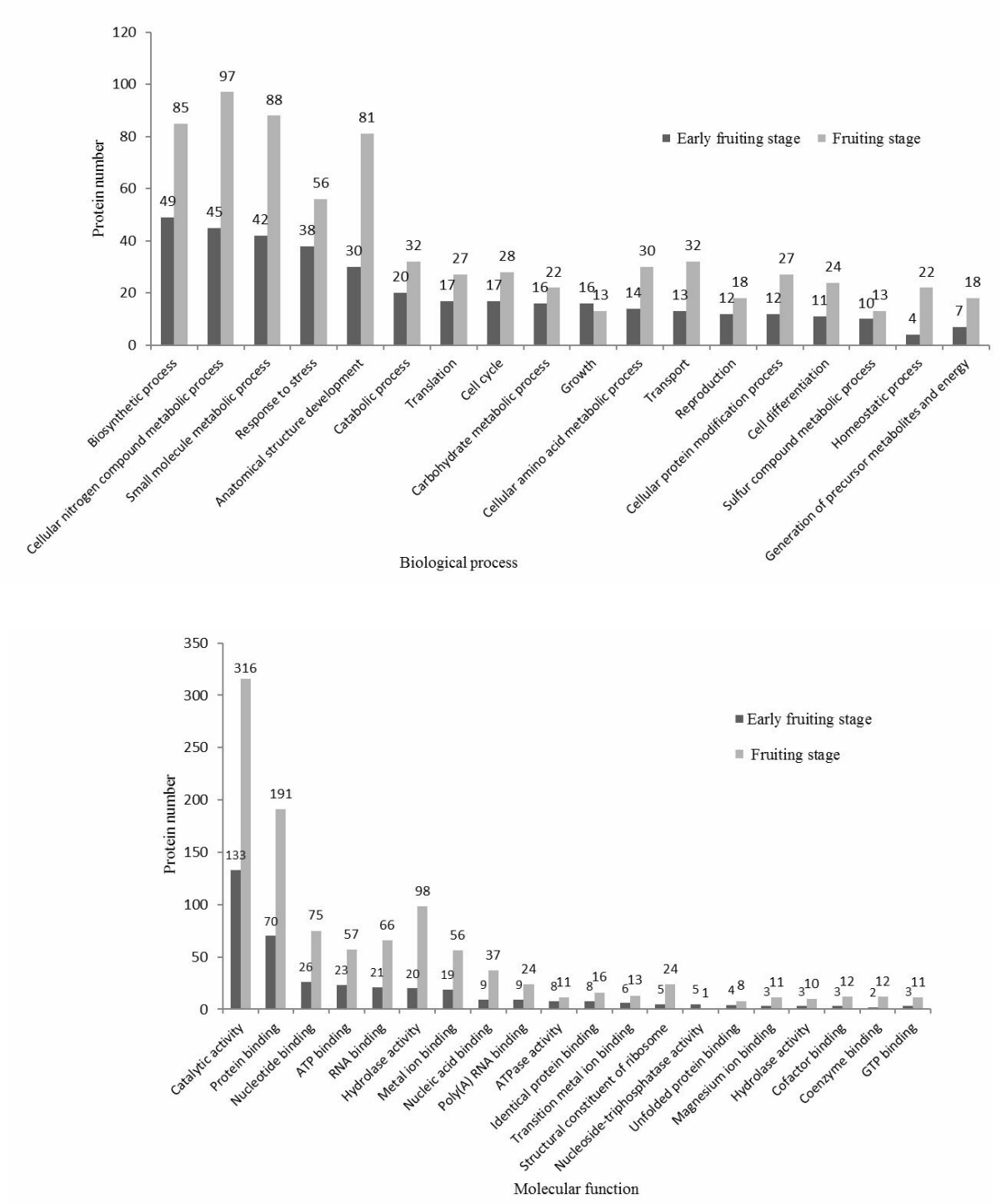

Figure 3. GO analysis of proteins expressed during the early fruiting and fruiting stages of *S. latifolia*.

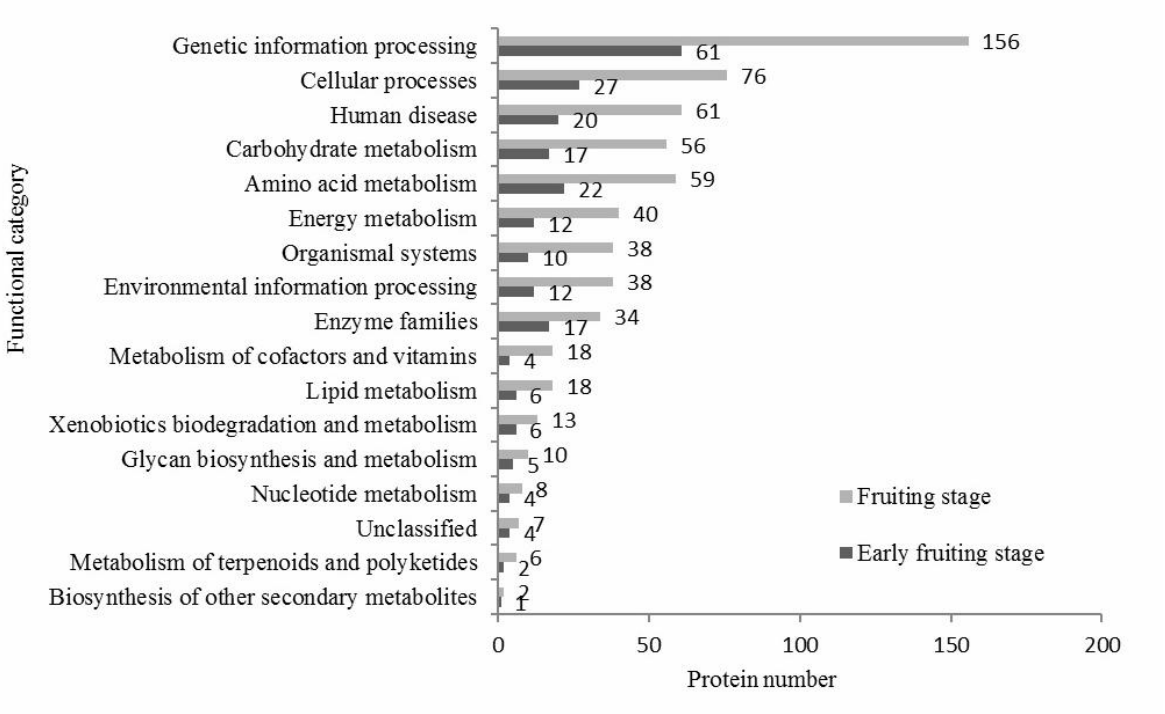

Figure 4. Functional category of proteins differentially expressed during the early fruiting and fruiting stages of *S. latifolia.*

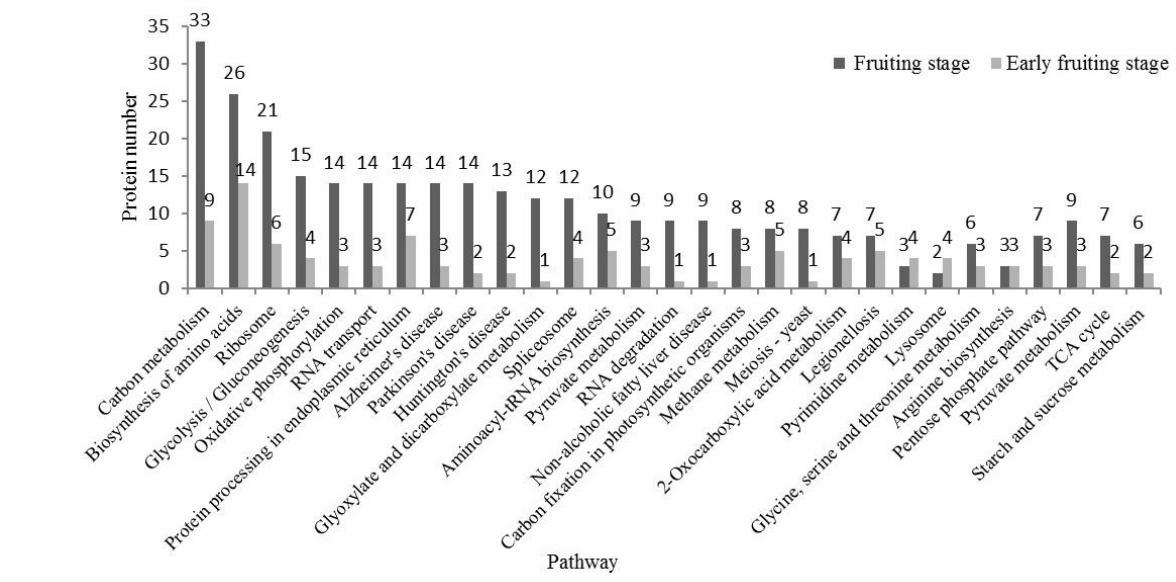

Figure 5. KEGG pathways of proteins differentially expressed during the early fruiting and fruiting stages of *S. latifolia.*

The Molecular mechanism of fruiting development

We obtained the mRNA sequence of *S. latifolia* aegerolysin-like gene namely *latifolysin* by transcriptome sequencing and analyzed it using bioinformatics methods. mRNA and protein expression were detected by Real-time quantitative PCR and Isobaric Tags for Relative and Absolute Quantification (iTRAQ) coupled two-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS), respectively. *Latifolysin* gene contained a 399 bp ORF that was predicted to encode a 133-ammino acid protein. The deduced latifolysin contained the structure domain of aegerolysin family, showing 40% identity to aegerolysin (Figure 6 and Figure 7). Fungal aegerolysin family proteins were inconsistently distributed among species. Phylogenetic analysis showed that latifolysin protein was clustered with basidimycete group and closely related to *Volvariella volvacea* (Figure 8). Quantitative real-time PCR and proteomics analysis revealed that the gene and protein of latifolysin had the highest expression level in the early fruiting stage and particularly higher than the mycelium stage (Figure 9). This study would be useful for understanding the developmental molecular mechanism of *S. latifolia*.

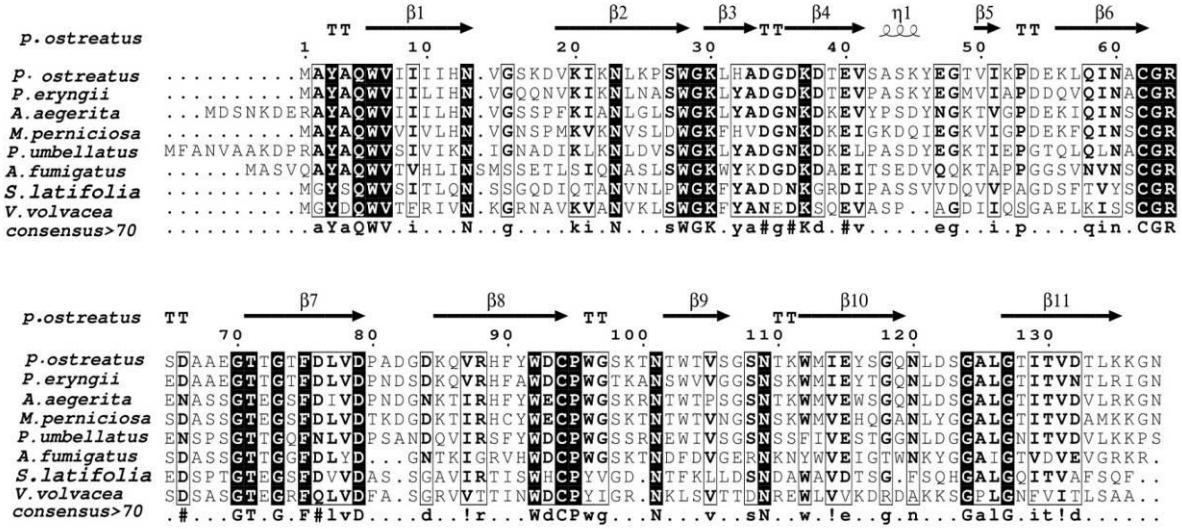

Figure 6. Cluster analysis of predicted amino acid sequences of latifolysin.

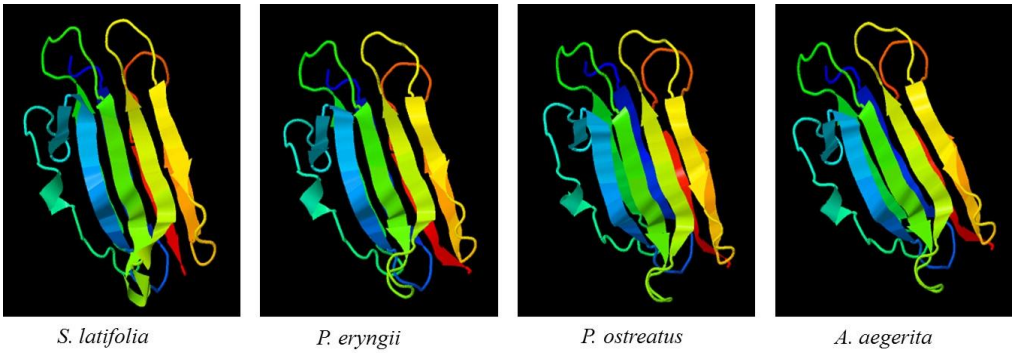

Figure 7. Predicted tertiary structures of hemolysin from four mushrooms.

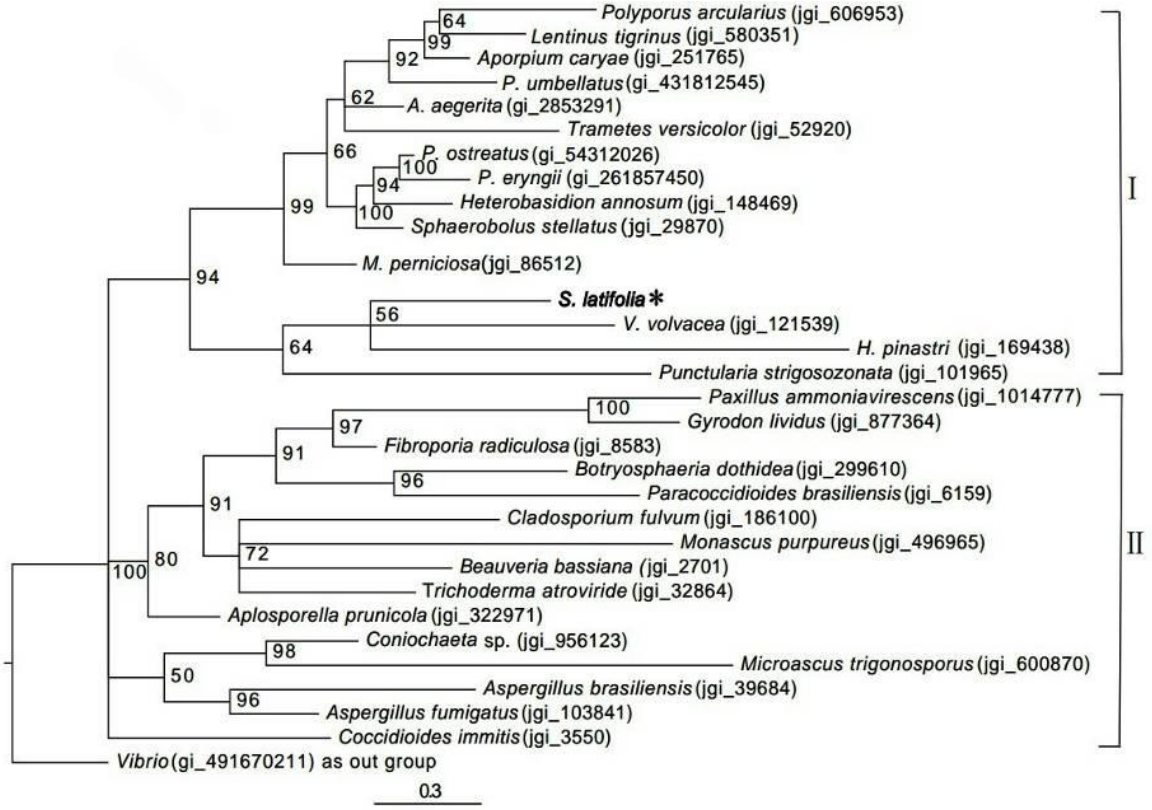

Figure 8. Phylogenetic analysis of some aegerolysins in fungi (*Vibrio* as outgroup).
Ⅰ: Basidiomycota; Ⅱ: Ascomycota

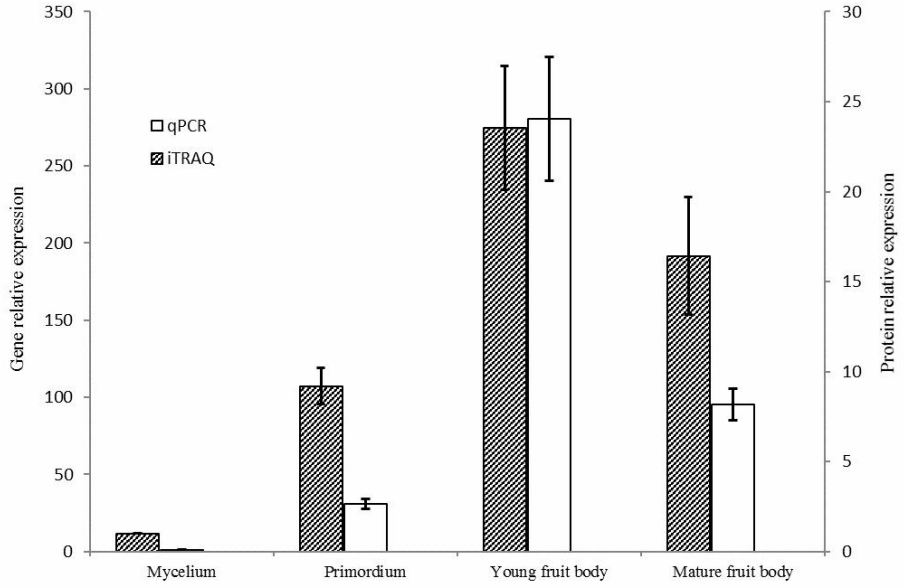

Figure 9. Differential expression of *latifolysin* gene at various developmental stages.

FACTORS AFFECTING *S. latifolia* GROWTH

Intrinsic factors

Carbon source

Carbohydrates are major components of the cytoskeleton, important nutritional requirements for growth and development (Xiao *et al.* 2006) and some of the most important required nutrients for *S. latifolia*. According to Shim *et al.* (1998), among 19 carbon sources, 16 carbon sources were favorable to the mycelial growth of *Sparassis crispa*. Except for salicin, adonitol and trehalose, mycelial growth of *S. crispa* was most favorable on culture media supplemented with maltose, followed by arabinose and mannitol. Huang *et al.* (2007b) found that highest growth rate and most luxuriant mycelia were observed when sticky rice powder served as the major carbon source. Vigorous growth was recorded on maltose, followed by glucose or sucrose, while no mycelial growth was observed when methylcellulose served as the carbon source. Our previous study showed that 27 g/L glucose was suitable for mycelium growth (Lin *et al.* 2011). Wang *et al.* (2012) also confirmed that starch was the main factor impacting the dry weight of mycelium. Farooq and Alfred Chioza (2014) reported that fructose followed by glucose supported the best mycelial growth while the most unsuitable carbon source was galactose (no mycelial growth). In liquid culture, You *et al.* (2006) found that starch and maltose were appropriate carbon sources for mycelial growth in submerged fermentation. Productivity of *S. crispa* mycelia reached its maximum at an initial glucose concentration of 30 g/L (Kurosumi *et al.* 2006). Our previous study showed that glucose was favorable for formation of small mycelial pellets and maximum biomass production was 3.53 g/L. Highest biomass (4.07 g/L) in medium was achieved when wheat flour was added in liquid culture, followed by glutinous rice flour and corn starch. These findings suggest that maltose, glucose and starchy carbon sources were suitable for mycelial growth of *S. latifolia*.

Nitrogen source

Shim *et al.* (1998) reported that glycine stimulated mycelial growth of *S. crispa* on culture media, but there was no mycelial growth on culture media that were supplemented with nitrogen sources such as methionine, glutamine, urea, histidine, ammonium acetate, calcium nitrate, sodium nitrate and potassium nitrate. You *et al.* (2006) showed that corn flour and bran were optimum nitrogen sources. Accord to Lin *et al.* (2007), *S. crispa* grew the best with culture media containing peptone, followed by vitriol ammonium and urea. PDA with 3 g/L peptone was suitable for mother-culture media. Huang *et al.* (2007b) also found that peptone, yeast extract or soybean meal was a favored nitrogen source. Farooq and Alfred Chioza (2014) found the most favorable nitrogen source was glycine followed by alanine and this is in agreement with findings of a previous study (Shim *et al.* 1998). In conclusion, organic nitrogen was more suitable for mycelial growth compared with inorganic nitrogen.

pH

Shim *et al.* (1998) reported that mycelial growth of *S. crispa* was most favorable at pH 4, whereas there was no mycelial growth at pH 8 and pH 9. Cheong *et al.* (2008) related that optimal pH for mycelial

growth was 6.0. According to Huang *et al.* (2007b), *S. crispa* mycelia grew over the full range of pH values tested (4.0-7.5), but the most suitable pH was 5.25 (highest mycelial growth rate). Slightly acidic to neutral initial agar media pH is most favorable for mycelial growth of *S. crispa* and PDA medium with initial pH of 6 resulted in highest mycelial growth among pH levels tested (Farooq and Alfred Chioza, 2014).

Substrate

Mushrooms can be classified into three categories by their tropic pattern: saprophytes, parasites or mycorrhizae. *S. latifolia* grows parasitically on stumps or roots of conifer trees during summer and autumn seasons (Kim *et al.* 2013). Lee *et al.* (2004) found that highest mycelial growth of *S. crispa* was recorded on larch sawdust. When *S. crispa* DUM-04 was cultured on a medium of larch sawdust + pine sawdust, formation of its basidiocarps was more outstanding on media containing larch/pine sawdust than those of only larch sawdust. Ryu *et al.* (2009) considered that *Larix kaempferi* was suitable for *S. crispa* cultivation including mycelial growth period, harvest period and mushroom production, respectively. Park *et al.* (2011) recommended that sawdust-based medium with larch for the cultivation of cauliflower mushroom be prepared with 0.76 g/cm^3 in medium density and excluding particles less than 1 mm. Researchers at FAAS have focused on *S. latifolia* artificial cultivation techniques since 2004 and successfully cultivated this mushroom in 2005. PDPA was the optimum mother culture medium of *S. crispa* while culture media from Mango and *Pinus massoniana* were suitable for *S. crispa* growth. Rice bran and corn powder were suitable complementary culture materials (Lin *et al.* 2005).

In conclusion, the most suitable substrate for mycelial growth and fruit body formation of *S. latifolia* is a mixture of larch sawdust and pine sawdust such as *Abies holophylla* (Ryu *et al.* 2009), *L. kaempferi* (Ryu *et al.* 2009), *Pinus koraiensis* (Oh *et al.* 2009), *Pinus densiflora* (Lee *et al.* 2004), *Larix gmelini* (Rupr.) Kuzenneva, *Larix olgensis* A. Henry and *Pinus yunnanensis* Franch (Liu 1986).

Extrinsic factors

Temperature

S. latifolia requires low temperatures in order to form fruit bodies. The range of temperature for mycelial growth of *S. latifolia* is 10～30℃ and the optimal temperature for *S. latifolia* mycelial growth is 23～24℃. *S. latifolia* mycelia stop growing above 30℃ and serious mycelial damage occurs at 30℃. Mycelium dies above 40℃ (Huang *et al.* 2007b). The optimal temperature for primordium formation was 20～21℃ (Huang *et al.* 2007a).

Humidity

Water is important for *S. latifolia* growth and production. Nutrients should be dissolved in water in order to be absorbed by the mycelium. The suitable moisture content of substrate is 60%～65% for vegetative growth of *S. latifolia*. Optimum humidity range of the incubation room is 85%～90% while during basidiocarp formation it is 90%～95%. (Huang *et al.* 2007a).

Air composition

S. latifolia is an aerobic fungus. Well-circulated fresh air and more frequent ventilation during the reproductive phase are important. According to our previous study, *S. latifolia* was very sensitive to carbon dioxide during primordium formation (Lin *et al.* 2007) and carbon dioxide levels were maintained below 0.03% (Zhu and He 2008).

Luminosity

Light is the direct or indirect source of energy for *S. latifolia* growth. Mycelium can grow in darkness without light, but light is required for basidiospore formation. *S. latifolia* needs much more light than other mushrooms and our previous study showed that fruit body formation was possible when the light was at 500～800 Lux.

ARTIFICIAL CULTIVATION

Selection of strains with high yield and good quality

Quality strains result in high yield and good quality mushrooms. Six strains of *S. crispa* were tested for their suitability for mycelial growth and primordium formation on various sawdust media in bottles. Strain DUM-04 showed good performance for fruit body formation. Agronomic characters of the fruit bodies were (average): 7.2 cm in height, 10 cm in diameter and the dry weight was 7 g (Lee *et al.* 2004). Ryu *et al.* (2009) investigated cultivation characteristics of 12 stains on *L. kaempferi* sawdust medium. Strain KFRI 700 showed the highest yield (163 g from 380 g sawdust media) with 103 days in the growth period. Features of the fruit body were: 14.9 cm in width and 8.5 cm in height.

Our team has artificially cultivated *S. latifolia* since 2005. We have investigated agronomic characters including biological characteristics, nutritional physiology, undesired microorganism resistance capacity and productivity. One strain, certified by the Variety Certification Committee of Fujian Province in April 2013, was formally registered as 'Min Xiu No.1'. This was China's first self-owned intellectual property variety. It was suitable for commercial cultivation with high biological efficiency and yield and harvest time at 150～200 g/bag and 120 d, respectively. Fresh *S. latifolia* fruit bodies had good shelf life and were hard and brittle with golden yellow color after drying.

Spawn making

In China, researchers tried to domesticate this mushroom early in the 1980s (Liu 1986), but little progress was made on artificial cultivation. FAAS has focused on *S. latifolia* artificial cultivation techniques since 2004 and has developed a culture medium (PDPA) for mycelium growth that contains 20% potato, 2.1% dextrose, 0.32% fish peptone and 2% agar (Lin *et al.* 2011). For *S. latifolia*, sawdust spawn is widely used

for its industrial cultivation because it is easy to manufacture without specific fermentation equipment. Mango and *Pinus massoniana* sawdust were suitable for *S. latifolia* mycelial growth (Lin *et al.* 2005). *S. latifolia* sawdust spawn was made using glass bottles (750 ml) containing *P. massoniana* sawdust (70%), corn flour (28%) and calcium carbonate (2%). After inoculation with mycelial plugs from PDPA slants, spawn was incubated at 25ºC in the dark until the substrate was completely colonized. According to our previous study, liquid spawn led to rapid mycelial growth on sterile pine sawdust substrate compared to traditional sawdust spawn (Ma *et al.* 2016). Further work on primordium development and fruit body formation by inoculating with liquid spawn is under investigation in our laboratory.

Substrate composition, spawn run and fructification

Korean scientists first artificially cultivated *S. latifolia* in 2004. Lee *et al.* (2004) found highest mycelial growth on larch sawdust, but larch + pine sawdust was more effective in forming fruit bodies than larch sawdust. Culture media included: *L. kaempferi* (60%), P. *densiflora* (20%), wheat bran (15%), and glucose (5%). According to Ryu *et al.* (2009), *S. crispa* strain KFRI 700 had the highest yield at 163 g from 380 g *L. kaempferi* sawdust media. Substrate used to cultivate *S. latifolia* contained pine sawdust (76%), wheat bran (18%), corn flour (2%), sucrose (2%) and calcium superphosphate (2%). The dry ingredients were thoroughly mixed and then tap water was added to reach a moisture content of 65%. A bag-filling machine was used to fill and compact substrate into polyethylene bags (17 cm in diameter and 36 cm long), a single, vertical hole was made in the center of the compacted substrate for the spawn inoculation and aeration. The bags were enclosed with plastic rings and vent caps, autoclaved at 122℃ for 2.5 h, then bags were cooled down to room temperature. The bags were inoculated with sawdust spawn (15±2 g) into the vertical hole of the substrate. Inoculated bags were incubated at 24±1℃ under dark conditions. After the substrate was completely colonized by the mycelia, the bags were then transferred into the fruiting chamber where the environmental conditions were maintained at 20～21℃, and 85 %～90% relative humidity, with a 10 h illumination. When primordial formed, the necks and covers were removed from the bags. Only a single flush of fruit bodies was harvested.

Cultivation

Until now, there are only few farms in Japan, Korea and China where this edible and medicinal mushroom is cultivated on an industrial scale. In the 1980s, researchers in Japan began to isolate wild strains of *S. latifolia* and initiated efforts to domesticate this mushroom including suitable culture media selection, identifying environment conditions for primordium formation, etc. In 1993, *S. latifolia* was successfully artificially cultivated. Bottle cultivation was realized in 1996. After 2000, artificially cultivated *S. latifolia* captured consumer interest in the Japan mushroom market. At present, there are some companies that cultivate this mushroom, including UNITIKA (Kwon *et al.* 2009, Yao *et al.* 2008, Yamamoto and Kimura 2010, Yamamoto *et al.* 2009, Yoshikawa *et al.* 2010, Yamamoto and Kimura 2013), Minahealth (Ohno *et al.* 2000, Harada *et al.* 2002a, b), etc., but cultivation techniques were not known to the public.

In order to shorten harvest time and improve yield performance, biological and cultivation characteristics of *S. latifolia* have been studied systematically by our research group. Huang *et al.* (2007a) and Jia *et al.* (2010) also reported a cultivation technique for *S. crispa*. Liu *et al.* (2010) found that *S. crispa* mycelial growth was rapid on pine sawdust after high temperature and pressure treatment and this process is now used for industrial cultivation.

Figure 10. Commercial cultivation factories of *S. latifolia* in China

FUTURE PERSPECTIVES

S. latifolia is an edible and medicinal mushroom that has been cultivated in recent years. The survival and multiplication of *S. latifolia* is related to a number of factors that may act individually or have interactive effects. A combination of these factors may have a complicated effect on production of *S. latifolia*. With intensive studies on its medicinal value, a number of new related products were successfully produced. In our country, research on *S. latifolia* developed late but is progressing rapidly mostly related to breeding, biological characterization, artificial cultivation, extraction of bioactive compounds, etc. Although we have made substantial progress additional work remains. Collection, conservation and domestication of germplasm resources of *S. latifolia* should receive more attention in the future. Breeding high yield, good quality and disease-resistant cultivars of *S. latifolia* that are suitable for industrial cultivation are crucial. And finally, bioactive compound extraction and its pharmacological action should also be emphasized.

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7. EFFECT OF SOAK TIME AFTER HARVEST ON NUTRITIONAL QUALITY OF *Tremella fuciformis* DRY PRODUCT

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ABSTRACT

This work examined the influence of soak time prior to drying *Tremella fuciformis* on sensory quality and nutrient composition. Soak time caused changes of pH of soak water, affecting color and tightness of the dry product. Soak time was linearly related to dry matter and ash content while changes of crude protein, crude fiber, crude fat and crude polysaccharide were less affected by soak time.

Keywords: *Tremella fuciformis*, soak time, nutritional quality, dry product

INTRODUCTION

Tremella fuciformis is an important edible fungus and is one of the historical treasures of China (Huang 2000). Wild *T. fuciformis* is widely distributed in China, but it is difficult to find specimens. At present, the vast majority of *T. fuciformis* is cultivated on sterilized substrate contained in bags (Peng *et al*. 2005). Gutian County of Fujian Province is the main producing area of *T. fuciformis* with an annual output of 30,000 tons, accounting for more than 90% of the national production of *T. fuciformis*. Products are exported to 27 countries and regions including Europe, United States and Southeast Asia. Because fresh fruit bodies of *T. fuciformis* do not have a long shelf life, they are usually stored and sold as dry product (Huang and Zheng 2009).

Before drying, freshly-picked *T. fuciformis* are soaked in water to remove debris and improve consumer appeal (Wang and Yang 2007). There is no uniform standard for soak time for *T. fuciformis* and soak water waste is a concern for local environments. Therefore, we began this work as commissioned by the Standardization Administration of China to examine the influence of soak time during pre-processing of *T. fuciformis* on sensory quality and nutrient composition. We sought to: 1) better understand the effect of soak time on quality, 2) assess the potential effect of discharge water on the environment and 3) develop relevant standards to guide production and processing of this mushroom.

MATERIALS AND METHODS

Materials

Fresh *T. fuciformis* fruit bodies produced in Gutian county were used as raw materials. Research was conducted at Yancang Edible Fungus Professional Cooperatives in Daqiao, Gutian county.

Test design

Fresh cut fruit bodies (40 kg) of *T. fuciformis* from the same producer were used for soak treatments (Table 1). Mushrooms for all treatments were completely immersed (soaked) in potable water in the same size containers. After soaking, mushrooms were placed on bamboo mats. Samples (0.5 kg) of fruiting bodies were randomly selected from each treatment after drying and evaluated for sensory quality and other parameters.

Table 1. Soak times for five treatments of *Tremella fuciformis* before drying.

Trt. No	Soak time (min)[a]
1	Not soaked
2	5
3	30
4	60
5	120

[a]Excess water was removed after soaking

Determination of indicators and methods

pH of soak water

After soaking, pH value of the water (23º C) was measured and 500 ml of soak water samples were taken. pH was measured again after standing 24 h and 5 days.

Sensory quality evaluation

After drying, shape, cleanliness and color of fruit bodies were estimated by sensory evaluation while diameter, height and other indicators were recorded.

Determination of nutrient composition

Analyses were made for moisture, crude protein, crude fat, crude fiber, crude polysaccharide, ash, colloidal protein, etc.

Figure 1. Fruit bodies of *Tremella fuciformis* after soaking and cleaning.

Figure 2. Fruit bodies of *Tremella fuciformis* after drying with hot air.

RESULTS AND ANALYSIS

Changes of pH value of water after soaking of *Tremella fuciformis*

The pH values of water collected after soaking tended to decrease with increasing soak time and increasing time of incubation. The pH value of Treatment 2 (5 min soak) was 6.7 while the pH value of water soak treatment of 30 min - 120 min decreased to 5.4 - 5.7 (Table 2). pH value of treatment 2 was 6.7, 6.0 and 5.2, immediately after soaking, at 24 h and 5 days, respectively. The pH value of water samples for Treatments 3 to 5 reduced by 0.2 - 0.4 with slightly acid water smell. After the water samples were kept in the environment at 25℃ for 5 days, the pH value of treatment 2 samples decreased from 6.0 to 5.2 and the water had a slightly acid odor. The pH value of the water samples of treatments 3 - 5 reduced to 4.2 - 5.0 and the water was malodorous. Odor ranged from normal water smell to weak acidity for Treatment 2 over the 5-day period (Table 2).

The influence of soak time on color and shape of *Tremella fuciformis*

Length of soaking time had a substantial influence on color and shape of *T. fuciformis* (Table 3). The color of non-soaked treatment 1 was the most yellow. The longer the fruit body was soaked the lighter the color. After 120 min soak time, dry fruit body color change was difficult to distinguish.

The shape of fruit bodies of treatment 1 was compact and tight. As soak time increased, the shape of the whole fruit body (treatments 2-5) became looser and the spread was larger. After 60 min soak time, fruit body shape tended to stabilize. Soak time did not appear to have much effect on the average height of the whole fruit body.

Table 2. pH value and "odor" of water after different soak times (not soaked or soak times)

Treatment No. (soak time)	Standing water time after soaking fruitbodies (pH/odor)		
	Water immediately after soaking	Water after standing for 24 h	Water after standing for 5 days
1 (not soaked)	-	-	-
2 (5 min)	6.7 Odor normal	6.0 Odor normal	5.2 Odor slightly acid
3 (30 min)	5.7 Odor normal	5.5 Odor slightly acid	5.0 Odor sour
4 (60 min)	5.6 Odor normal	5.2 Odor slightly acid	4.4 Odor sour
5 (120 min)	5.4 Odor normal	5.3 Odor slightly acid	4.5 Odor sour

Table 3. Influence of soak time on color and shape of *T. fuciformis*

No.	Fresh weight（kg）	Color[1]	Tightness[2]	The average diameter（cm)[3]	The average height（cm)[3]	Dry weight（kg）
1	4	Yellow 1	Tightness - 5	10.8	5.8	1.30
2	4	Yellow-2	Tightness - 4	12.9	6.0	1.28
3	4	Yellow-3	Tightness -3	13.5	6.0	1.30
4	4	Yellow-4	Tightness - 2	13.6	5.6	1.28
5	4	Yellow-5	Tightness -1	13.4	6.0	1.25

Note：

[1] Yellow 1-5, where 1= darker yellow and 5= lighter yellow.

[2]Tightness 1-5, where 1=slightly tight and 5=tightest

[3]Average measurement of 10 random samples each.

Influence of different soaking time on nutritional components of *Tremella fuciformis*

The comparison of nutrients as affected by soak time is presented in Table 4. It is observed there that the water content of each treatment of dry product of *T. fuciformis* was less than 15%, according to the national standards, they meet the water content requirements of dry products. Also, the nutrient contents after *T. fuciformis* drying is presented at different water soaking times (Table 5).

Table 4. Comparison of nutritional components of *Tremella fuciformis* as affected by soak time

No.	Moisture %	Ash%	Crude protein %	Crude fiber %	Crude fat%	Crude polysaccharide %	Collagen
1	11.8	7.9	8.8	2.4	1.8	9.0	not detected
2	11.1	7.8	10.0	2.2	2.6	6.6	not detected
3	13.8	6.8	10.1	2.6	2.0	10.0	not detected
4	13.7	5.8	10.2	2.6	2.3	7.8	not detected
5	12.4	6.2	10.0	2.3	2.2	9.7	not detected

Table 5. Nutrient content of dried *Tremella fuciformis* after different soaking time

Unit: kg

No.	water content %	Ash%	Crude protein %	Crude fiber %	Crude fat%	Crude polysaccharide %	Collagen
1	1.147	0.091	0.101	0.028	0.021	0.103	0
2	1.138	0.089	0.113	0.025	0.03	0.075	0
3	1.121	0.076	0.113	0.029	0.022	0.112	0
4	1.105	0.064	0.113	0.029	0.025	0.086	0
5	1.095	0.068	0.11	0.025	0.024	0.106	0

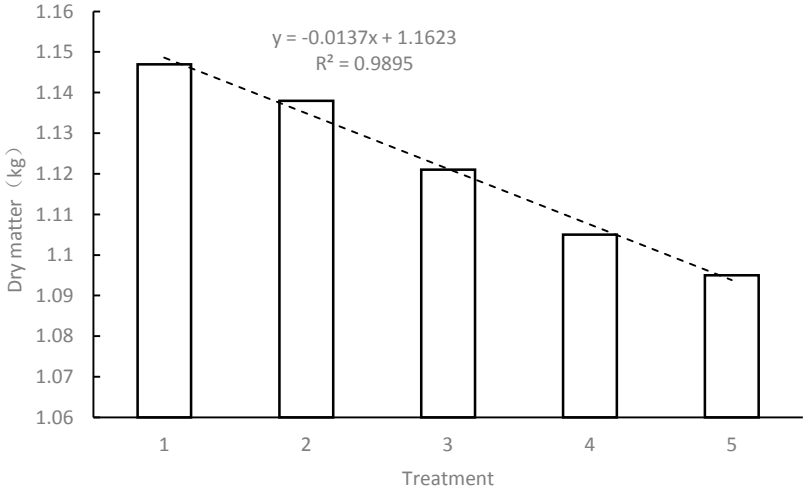

Fig.3 Column chart of dry matter after different treatment

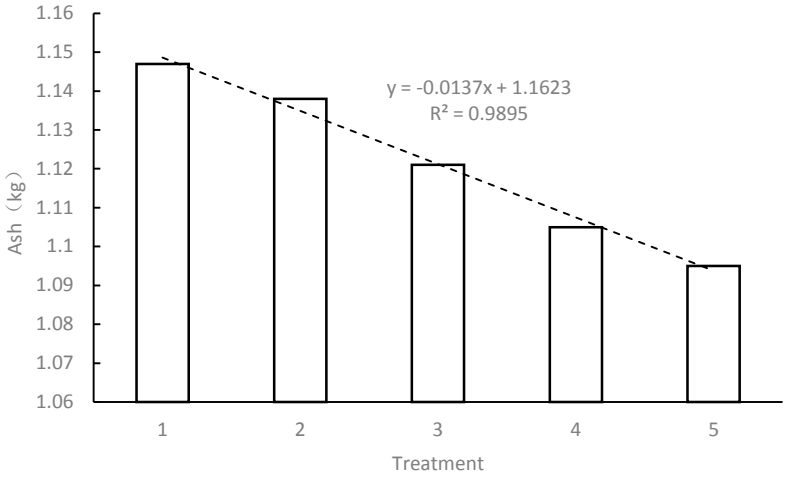

Fig.4 Column chart of ash after different treatment

As soak time increased, total weight and ash of dry products decreased - this trend was linear (Table 4, Figs 3-4). The longer *T. fuciformis* absorbed water the more substances were lost. Highest loss of dry substances and ash occurred in Treatment 5. Although there were differences in aspects of crude protein, crude fiber, crude fat and crude polysaccharide, depending on treatment, no apparent linear changes were observed. Therefore, we concluded that the changes of crude protein, crude fiber, crude fat and crude polysaccharide were not related to soak time.

DISCUSSION

As soak time increased, the pH of soak water gradually decreased. The reason may be that nutrient substances of *T. fuciformis* fruit bodies were dissolved into the water. These substances would provide nutrients for bacterial growth and reproduction and thus, cause water rancidity. Therefore, freshness of soak water should be monitored in real-time and changed in time to reduce microbial growth. Soak time significantly affected the sensory quality of *T. fuciformis*, such as color and shape, and appears to cause the loss of the nutrient substances. Therefore, soak time of *T. fuciformis* should be well controlled under practical operation, to avoid a drop in nutritional value (Huang *et al*. 2009).

Changes in contents of crude protein, crude fiber, crude fat and crude polysaccharide were slightly affected by length of soak time. Protein components are just part of the fruit body and free state protein components were not lost. Soak water used in these experiments was well water with normal temperature. Therefore, degeneration, decomposition and precipitation of protein components were minimized. Crude fiber is the main portion of the cell wall of the fruit body and since it is not soluble, it remained intact. Most of polysaccharides were not dissolvable, so the soaking time would not cause the loss of polysaccharides. Most fungal polysaccharides are insoluble in water so soak time does not affect polysaccharides content (Chen

2011). Polysaccharide content of *T. fuciformis* was not affected by soak time.

After cooking, the soup of *T. fuciformis* is "sticky" while the fruit body is gelatinous. Many consumers believe *T. fuciformis* is rich in gelatin, which is considered to be collagen. Collagen is nutritional complement for anti-aging and exists in the animal body. Hydroxyproline is the characteristic amino acids of collagen. Collagen was not detected in the 5 samples tested. There are two possibilities. Firstly, there is no collagen in the fruit body of *T. fuciformis*. Secondly the content of collagen of the fruit body of *T. fuciformis* is quite low and can't be detected (Huang *et al.* 2010, Liu *et al.* 2006). The minimum detection volume was 0.1mg/100 g. The materials we studied show that the fruit body of *T. fuciformis* doesn't contain this kind of protein. Therefore, it is presumed that *T. fuciformis* does not contain collagen. The gelatin of the fruit body of *T. fuciformis* is a kind of mucopolysaccharide that is rich in glucose, trehalose, acid pentose, mannitol, etc. Mucopolysaccharide is the essential substance of the cell cytomembrane, similar in structure to hyaluronic acid with excellent moisturizing properties (Yan *et al.* 2006).

It is assumed that the length of soak time mainly depends on consumer and market demand of the shape, looseness and color of *T. fuciformis*. Meanwhile, it is necessary to correctly guide sales of *T. fuciformis* in order to make sure quality and nutrition are considered first followed by appearance.

ACKNOWLEDGMENTS

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8 FRUIT BODY PRODUCTION OF *Schizophyllum commune*

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ABSTRACT

Cultivation of tropical edible mushrooms has generated an enormous and growing interest because it represents a new alternative for various sectors to produce beneficial food products using environmentally friendly biotechnologies. In particular, the academic sector has prompted development of research to diversify means and techniques of cultivation while also integrating various aspects such as traditional knowledge and use of local genetic resources. In this chapter, data on biology and consumption of *Schizophyllum commune* Fr. in tropical regions are presented in addition to observations on cultivation of this fungus under semi-controlled conditions on several agricultural by-products from Tabasco, México.

Keywords: edible mushrooms, basidiomycetes, mushroom cultivation, fungal technology

INTRODUCTION

In recent years, efforts have increased in Latin America to integrally understand diversity of macroscopic fungi in different environments. Edible and medicinal mushrooms have been catalogued in micro-floristic studies and their sustainable cultivation on agricultural by-products, which presents multiple benefits for both humans and the environment. However, in spite of being a relatively accessible activity with a growing tendency, knowledge on fungi cultivation in tropical areas has been generated to a lesser extent as a result of multiple factors.

Since substantial research exists on edible mushrooms and their cultivation in temperate climates but not tropical climates, one strategy has been to develop sustainable production of commercial strains with the capacity to produce fruit bodies at an average ambient temperature of 30 °C. This strategy was based on experiments and improvements in the laboratory as well as in conditioned cultivation chambers, in addition to considering other technical aspects. In this way, greater knowledge on fungi production, including appropriate conditions and culture practices, has been generated. Even so, the majority of commercial species optimally produce fruit bodies at an average temperature of 20 °C (Marshall and Nair 2009).

Another cultivation strategy has focused on the use of available genetic resources in the region, employing native strains that are adapted to conditions of a tropical climate in addition to developing various cultivation processes and techniques that include substrate management and that also consider the economic and cultural characteristics of local populations. With this strategy, traditional knowledge has formed a fundamental part of the development of cultivation techniques and related research on this theme has undoubtedly increased the growth opportunities for mushroom cultivation.

In Mexico, several fungal species are cultivated using imported strains, such as button [*Agaricus bisporus* (J.E. Lange) Imbach] and oyster mushrooms [*Pleurotus ostreatus* (Jacq. ex Fr.) Kumm] that are

merchandized in supermarkets, street markets and small fruit and vegetable shops, mainly in the central and southern regions of the country. However, at least 371 wild species of edible mushrooms have been identified (Garibay and Ruan 2014). At least one dozen of these may be distinguished for their important ties to local and indigenous cultures and are currently widely accepted and consumed amongst rural populations of temperate and tropical zones. Given the biology of these species as facultative saprophytes, they are ideal candidates for experimentation in order to examine their cultivation potential. Amongst these tropical edible species, many fungi belonging to the genus *Pleurotus, Hydnopolyporus*, *Lentinus*, *Auricularia*, *Polyporus* and *Schizophyllum* are found (Moreno-Fuentes 2014).

Especially, in the state of Tabasco, *Auricularia delicata* (Fr.) Henn, *A. polytricha* (Mont.) Sacc., *Pleurotus djamor* (Rumph. ex Fr) Boedijn and *Schizophyllum commune* are traditionally eaten, mainly in the municipalities of Macuspana and Teapa. The frequent sale and cultural importance of *S. commune* have also been highlighted. In this regard, research on the management of strains of these species and their production is of great importance, with the goal of developing a technical guide to facilitate production of these fungi for diverse sectors of the population.

At the Universidad Juárez Autónoma de Tabasco (UJAT), studies have been carried out since 2012 to obtain and characterize native strains of the aforementioned species. Comparative tests of mycelial growth were performed *in vitro* using various substrates, such as banana leaves (*Musa paradisiaca*), cacao shells (*Theobroma cacao*), coconut fibre (*Cocos nucifera*) and cedar sawdust (*Cedrela odorata*) (Carreño-Ruiz *et al*. 2014).

Schizophyllum commune is a species, that due to its medicinal and gastronomic properties, as well as its resistance to high temperatures has generated an interest in the continuity of these studies realized in the UJAT to optimize its production. One of the advantages of *S. commune* is its cartilaginous texture, which compared with other species of fleshy edible fungi, is more resistant to decomposition in tropical climates. In this chapter, experiences in cultivating this species using agricultural by-products of the region are described.

BIOLOGY AND CULTIVATION OF *Schizophyllum commune*

Several studies have shown that *S. commune* has a wide distribution around the world, except in Antarctica (Chang and Lui 1969, Adejoye *et al*. 2007) and grows throughout the year (Degreef *et al*. 1997). This fungus is found on trunks of fallen trees, on recently cut branches, on dead wood and on more than 300 live plant hosts (Vázquez-Mendoza 2012). It develops in areas covered with vegetation or in grasslands, can survive during the dry season or on wood exposed to sun (Mata 1999) and leads to the white rotting of wood. It has been found growing in numerous types of vegetation like tropical and subtropical forests, tropical montane cloud forests, *Quercus, Pinus* and *Abies* forests, thorn forests, xerophytic scrub, grasslands, coastal and urban vegetation and fruit crops (Olivo-Aranda and Herrera 1994). In the state of Tabasco, it has been found on banana pseudostems, fallen wood of 'palo mulato' (*Bursera simaruba*), cacao shells and coconut fibre, amongst other substrates.

This species is characterised by a flabelliform or fan-shaped fruit body of 7 to 25 mm in width and 10 to 30 mm from the base toward the outer edge (Figure 1 A). It presents sessile to substipitate pilei with irregular edges that are leathery in texture. The pileus is greyish white (identified with the key $N_{10}M_{00}C_{00}$ according to the Color Guide of Küppers 1996) and the upper surface is hairy. The hymenium presents longitudinally split gills (lamella) (Figure 1 B) that are light brown in color ($N_{00}A_{10}M_{00}$-Kuppers 1996) (Carreño- Ruiz *et al*. 2014).

Other morphological forms of the species also are found. For example, sessile spatulate or with psuedostipitates or with semi-circular and connate forms. Varying sizes and colors are also found, depending on the state of development of basidiocarps, availability of nutrients and environmental conditions.

Schizophyllum commune has long generated human interest and is recognised by various ethnic groups and common names in distinct languages. Initial records have shown that this fungus was both eaten and used as a type of chewing gum (Cooke 1961).

More recently, several research studies were carried out highlighting the consumption of this species in countries such as Mexico (Olivo-Aranda and Herrera 1994), India (Longvah and Deosthale 1998), Japan (Paulí 2000), Nigeria (Adejoye *et al.* 2007), Guatemala (Bran-González *et al*. 2009), the Philippines (Reyes *et al.* 2013), Malaysia, Thailand (Mirfat 2014) and Argentina (Figlas *et al*. 2014). Related research studies have been initiated to obtain native strains, to examine mycelial growth and to evaluate the fruiting process of this fungus. In addition, Boa (2005) highlights the consumption of this species in other countries such as Benin, China, Ethiopia, Ghana, Hong Kong, Madagascar, Malawi, Peru, the Central African Republic, Laos and Zambia, where, in addition to being considered a food resource, it has also been used in traditional medicine.

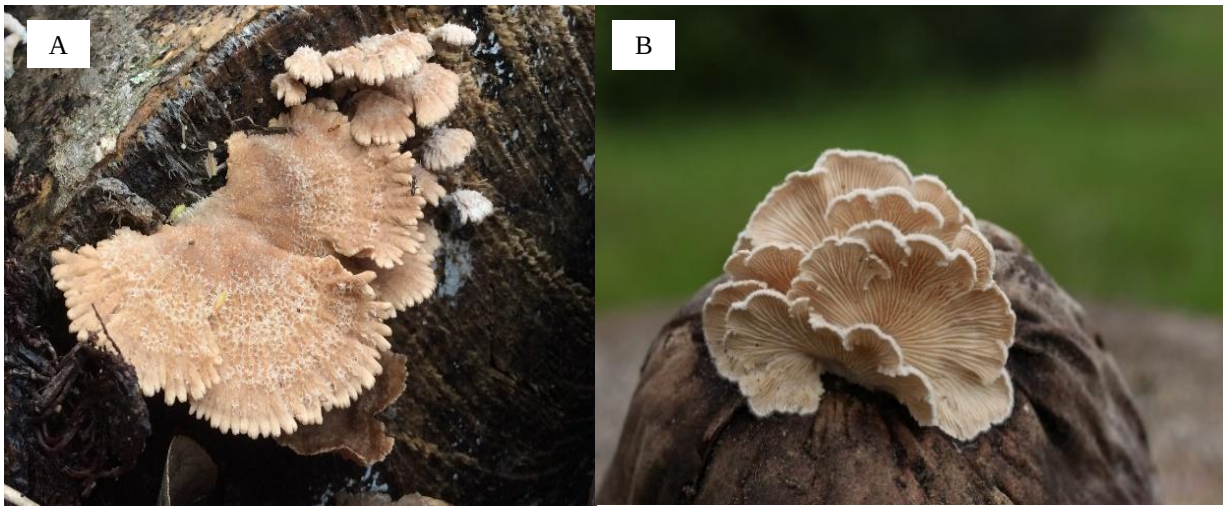

Figure 1. A) Flabelliform basidiocarps of *Schizophyllum commune* (photo by Mario Eduardo Sosa).
B) Lamellae of the hymenium in detail (photo by Víctor Herman Gómez García).

In Mexico, *S. commune* is reported in nearly all states. However, its consumption has mainly been recorded in states of the south-eastern region, such as Veracruz (where it is known as 'chiquite' or 'hongo de chaca'), Puebla ('oreja de ratón'), Oaxaca ('hongo blanco'), Chiapas ('xikin che', 'afumaditos', 'cascarilla de madera', 'pajarito', 'sulte' and 'tehuitzhanacame'), Tabasco ('oreja de palo', 'cusuche', 'muca' and 'orejita criolla') and Quintana Roo ('oreja de palo') (Guzmán 1990, Guzmán 2003, Ruan-Soto *et al*. 2004, Ruan-Soto *et al*. 2006, Ruan-Soto *et al*. 2009, Ruan-Soto and Cifuentes-Blanco 2011, Vázquez-Mendoza 2012).

In the coastal plains of the Gulf of Mexico, this species is frequently sold and is also abundant in some traditional markets in Oaxaca and Tabasco. Sellers are largely indigenous women who commercialise either small sacks or larger quantities, demonstrating the wide presence and consumption of this species in the southern region of the country (Ruan-Soto *et al.* 2006).

In regard to its preparation, in Teapa, Tabasco, *S. commune* is used to make several traditional dishes, such as the so-called 'mone' (Ruan-Soto *et al.* 2006, Ruan-Soto and Cifuentes Blanco 2011), amongst others. In the Northern Sierra of Puebla, this species is used in soups or as a slight stimulant (Vázquez-Mendoza 2012).

Generally, the sale of *S. commune* occurs during the rainy season. Both sellers and consumers have demonstrated preference for larger fruit bodies of white color, although the diverse morphologies and variations of this species are also sold in markets or collected for self-consumption (Figure 2).

Figure 2. A) Sale of *S. commune* along with other regional products in the Diana Córdova de Balboa market in Teapa, Tabasco. B) Presentation of *S. commune* along with suggested ingredients for its preparation (photos by Santa Dolores Carreño Ruiz).

With respect to the nutritional properties of *S. commune*, studies have found a protein content of 16% to 27% (Aletor 1995, Longvah and Deosthale 1998), a low-fat content of 2% (20 g/kg in dry weight) and abundant oleic and linoleic fatty acids. The fungus also contains 34% of amino acids essential to the human diet (Longvah and Deosthale 1998).

In addition, *S. commune* possess medicinal properties, such as antibiotic, antitumor, antioxidant and anticarcinogen properties, highlighting its pharmacological importance. This fungus produces immunostimulant polysaccharides, schizophyllan or sonifilan ((1 3)-β-D-glucans with ramifications (1 6)-β-D-glucosyl) that are widely used for treatment of cervical cancer, mainly in Asian countries (Ooi and Liu 2000, Chang and Miles 2004). In traditional Chinese medicine, *S. commune* is used in the form of infusions to treat leucorrhoea (Ying *et al*. 1987, Chang and Miles 2004, Hobbs 2005, Adejoye *et al*. 2007, Calonge 2011, Vázquez-Mendoza 2012) and has also been used for regulating blood pressure (Boa 2005).

With respect to its cultivation, *S. commune* has been little studied at the international level for production purposes (Chang and Miles 2004). To date, methods to produce its fruit bodies were developed in Guatemala by Bran-González *et al.* (2009), who obtained basidiocarps of this species on various substrates, including pine shavings, dry coffee pulp, maize cobs and stalks, and oat and wheat flours. This

latter study established parameters for their optimal growth and development in this region and also experimentally studied different parameters in the production of fruit bodies.

Meanwhile, Figlas *et al*. (2014) evaluated growth of *S. commune* on sunflower seed residues from the oil industry in Argentina, suggesting that discarded sunflower shells may be used as a nutrition source and as a substrate for the cultivation of this species on synthetic trunks. This technique, in addition to substrate supplementation with wheat bran, was shown to significantly improve basidiocarp production.

Important and complementary studies exist on mycelial growth of *S.* commune on various culture media and substrates at average temperatures ranging from 26 to 30°C. These studies contain valuable references for the adequate isolation, conservation and management of the strains (Adejoye *et al*. 2007, Bran-González *et al*. 2009, Teoh and Don 2012, Figlas *et al*. 2014, Carreño-Ruiz *et al*. 2014).

CULTIVATION OF *Schizophyllum commune* IN TABASCO

In 2012, a collection of edible macroscopic mushrooms was started in the Tropical Mycology Laboratory of the UJAT (Cepario Cappello-García-CCG). To date, twelve strains of different species have been collected with five corresponding to the genus *Schizophyllum*. Several of these strains were deposited in the mushroom collection of the Instituto de Ecología, A.C. INECOL. Xalapa, México (Cepario de Hongos comestibles IE) (World Data Centre for Microorganisms) (Figure 3).

Figure 3. General aspect of the culture *Schyzophyllum commune* strain CCG003 on PDA medium (photograph by Santa Dolores Carreño Ruiz).

For fruiting experiments of *S. commune*, mainly cacao shells and coconut fibre were used. These substrates are collected fresh and then cleaned and sun dried for approximately five days. Afterwards, the cacao shells are broken into pieces of 2 to 3 cm^2 and the coconut fibres into strips of 5 cm in length. Then, the substrates are hydrated for 12 h. Excess water was subsequently drained until the approximate moisture content of 70% was reached, and 0.1% lime and gypsum were added. A thermal treatment was performed by immersing the substrate, contained in a metal recipient, in water at 80 °C for 1 h. Before inoculation, the substrate was drained and cooled.

The cultivation of *S. commune* was performed in a small greenhouse under semicontrolled conditions using a system of hanging bags (Figure 4 A) in which plastic, transparent bags of 35 x 45 cm were hung after manually being filled with alternating layers of the primary inoculum and substrate until reaching 1 kg (wet weight). Primary inoculum was grown on popcorn kernels (*Zea mays* L. var. *everta*).

The incubation period was under conditions of darkness during 16 days at ambient temperature. On the following day of inoculation, 35 perforations were made with dissecting needles and radially distributed to favor oxygenation. Initially, mycelium aggregates (joint of hyphae) appeared at the zones of perforation after approximately 12 days of incubation (Figure 4 B). When the mycelium had covered the substrate, bags were exposed to light in order to stimulate formation of primordia. And to allow for adequate development of fruit bodies, in this period cuts were then made in the form of an 'X' on the plastic bag and daily watering was performed to maintain a relative humidity of 75% to 85%. After maturation, fruit bodies were harvested (Figure 4 C).

Figure 4. A) Incubation of *S. commune* cultures under conditions of darkness using a system of hanging bags. B) mycelium aggregates during the incubation period and C) formation of primordia on the substrate (coconut fibre) (photographs by Santa Dolores Carreño Ruiz).

Biological efficiency, yield and rate of production (Gaitán-Hernández *et al*. 2006) were obtained. Likewise, periods of incubation and appearance of fruit bodies were recorded, including variables such as temperature, total harvest and size and fresh weight of fruit bodies. The above data were based on a single crop.

Production of fresh fruit bodies of *S. commune* on cacao shells and coconut fibres encompasses a cycle of approximately 47 days from time of inoculation. The appearance of primordia occurs two days after exposing the samples to light. Four days after, the harvest period is initiated for fungi grown on cacao shells, whilst the harvest of fungi grown on coconut fibres can vary by one day. Similarly, duration of the fruiting period is approximately 23 days.

These time periods are similar to those reported by other authors such as Bran-González *et al*. (2009) who reported that the cultivation of *S. commune* on maize stalks and corncobs (1:1) had a duration of 28 to 48 days, including the time lapsed from inoculation to the harvest of basidiocarps. Figlas *et al*. (2014) recorded a cultivation cycle of 36 days and a reduction to 31 days using supplemented sunflower seeds.

With respect to biological efficiency of substrates containing cacao shells and coconut fibres, values of 12.35% and 7.7% were obtained, respectively, which were slightly higher than the biological efficiency (5.5%) reported by Bran-González *et al*. (2009) for a Guatemalan strain of *S. commune* cultivated on maize stalks and cobs (1:1). Figlas *et al*. (2014) cited a higher value of biological efficiency (48.3%) using supplemented sunflower seeds in studies carried out in Argentina.

With respect to morphology of fruit bodies, flabelliform pilei have been observed (Figure 5 A) in addition to connate formations (Figure 5 B), with an average size of 9 to 40 mm and 15 to 50 mm in diameter, respectively. Larger sizes have been recorded for basidiocarps harvested on cacao shells.

During the harvest period, temperatures of 28 to 40°C were reported. Greater production was obtained with temperatures surpassing 30°C, demonstrating the resistance of *S. commune* to relatively high temperatures.

Contamination by bacteria or other fungi has not proven a problem. Although it is indispensable to disinfect the substrate to maintain aseptic conditions during culture, this species shows resistance to other microorganisms, which could be related to its antibacterial and antifungal properties. Studies on production of metabolites by *S. commune* have confirmed the effective medicinal properties of this fungus (Mirfat *et al*. 2014). However, it is essential to maintain daily cleaning of the greenhouse or production area.

Other important factors for development of fruit bodies are moisture content of the substrate and the environment, given that high temperatures can cause a decrease in their ability to retain moisture. Even so, *S. commune* can adapt to relatively dry environments and can resume growth and development when humidity conditions become more favourable. Cooke (1961) and Vellinga (2013) have confirmed that, after loss of humidity, the fruiting bodies of this fungus are able to return to their initial conditions following a recovery in humidity levels.

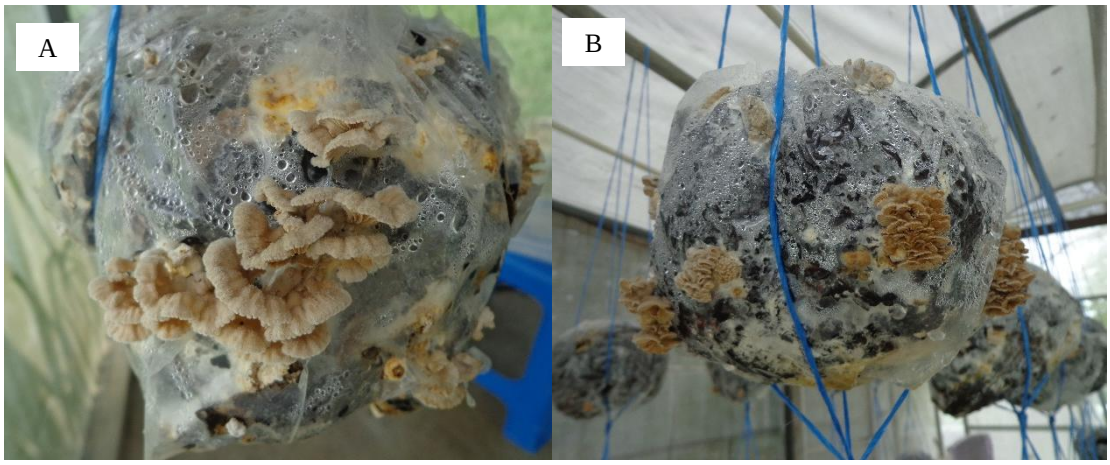

Figure 5. Shape patterns of pilei in *Schizophyllum commune* cultivation. A) Flabelliform pilei and B) connate pilei on cacao shells (photographs by Santa Dolores Carreño Ruiz).

PERSPECTIVES AND RECOMMENDATIONS

In Tabasco, the quantity of residues generated by the most intensive agricultural activities is estimated at approximately 16,000 t per year. Cacao and coconut production generate approximately 4,000 t of

residues in the form of fibrous shells (SIAP, 2016). Currently, these latter substrates are not reused and therefore could represent a valuable resource for *S. commune* production, as fruiting bodies occur naturally on these substrates.

Several aspects of *S. commune* production require further exploration. Evaluation of other substrates in addition to those previously mentioned, both alone or as a mixture, and the effects of fermentation processes should also be investigated. Despite the documented information on cultivation of this mushroom, the role of incubation systems and the appropriate conditions for cultivation rooms constitute other fundamental topics of study in tropical regions. In this sense, the optimisation of production and, in particular, the production of certain fruit body morphologies represents other challenges to address. Currently, at the UJAT, considerable interest lies in evaluating cultivation of this species under distinct production schemes, for example, in systems exposed to the environment that utilise other substrates such as sugarcane bagasse (*Saccharum officinarum*) (Figure 6 A) or different types of wood shavings (Figure 6 B).

This experience may be added to efforts to incentivise cultivation of *S. commune* in tropical regions. Culture techniques should be improved to achieve greater biological efficiency and to develop additional aspects of production. Production capacity of this species under semi-controlled conditions as well as its resistance to high temperatures may be highlighted as important qualities.

Furthermore, the improvement of culture techniques is indispensable in order to maintain the accessibility and viability of *S. commune* production for interested growers and to promote its consumption in the rural sector as a strategy for mitigating the lack of highly nutritional foods. In addition, developed culture systems can have multiple benefits for rural environments, such as, for example, the use of agricultural by-products.

A special point of interest is to keep studying this fungus, since there have been some reports indicating that under certain conditions it can be considered as pathogen for humans, causing mainly infections (Rihs *et al*. 1996, Kamei *et al*. 1999).

Figure 6. A) Fruit bodies of *S. commune* on sugarcane bagasse and B) wood shavings (photographs by Silvia Cappello García).

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9. BASIDIOME PRODUCTION OF GUATEMALAN STRAINS OF *Lepista nuda*

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ABSTRACT

This study determined biological efficiency and pileus diameter of basidiomes of five native strains of *Lepista nuda* on three substrates. Highest biological efficiency was 57.1% from a substrate formulated with 75% wheat straw and 25% rice husk (strain 21.10). Strains 17.01 and 50.09 fruited only on compost used for *Agaricus* cultivation. Largest pileus diameter (6.19 cm) was obtained for strain 21.10.

Key words: edible mushrooms, substrate, biological efficiency.

INTRODUCTION

Lepista nuda (Bull.) Cooke is consumed in several countries of Europe, America and Australia (Stott *et al.* 1996). This mushroom has good potential for production using the *A. bisporus* cultivation system because both species show similar patterns of growth (Danai *et al.* 2008). The origin of *L. nuda* cultivation is in Europe, but there are complications in production due to a slow and prolonged life cycle (Castro *et al.* 2014). Several studies were conducted in Australia, Israel and Spain that evaluated production of fruit bodies on different substrates. However, yield was relatively low and further experimental studies were recommended before attempting its commercial cultivation (Stott *et al.* 1996, Danai *et al*. 2008, Castro *et al.* 2014).

In Guatemala, *L. nuda* is used as food by the Maya-Kaqchikel people in the highlands of Chimaltenango (Morales 2001, Bran *et al.* 2003a, Bran *et al.* 2003b, Morales *et al*. 2010) (Figure 1). Due to the saprobic nature of this species, it can be cultivated on wastes that are generated from agricultural activities in the country. Several native strains have been evaluated on various culture media and temperatures. In addition, production of spawn on various grains and the development of primordia on two substrates have been accomplished. Given the progress that has been made for the cultivation of Guatemalan strains, it is desirable to evaluate production of fruit bodies (Bran *et al.* 2011, Bran *et al*. 2015).

The objective of this study was to determine biological efficiency (BE) and pileus diameter of basidiomes (fruit bodies) of Guatemalan strains of *L. nuda* produced on various substrates. Our work aims to: 1) develop an appropriate technology for cultivation of *L. nuda* in Guatemala, 2) serve as a preliminary step of cultivation technology transfer to rural communities, 3) provide the possibility of an alternative food, 4) assist in economic development of the country, and 5) serve as a bioprospecting model of fungal diversity.


Figure 1. *L. nuda* in Tecpán-Guatemala, Chimaltenango, Guatemala. Left: Wild fruit bodies on leaf litter in *Quercus*-dominated forest. Right: Mushrooms for sale at 94 Km Interamerican highway, including *L. nuda, Amanita garabitoana, Chroogomphus jamaicensis* and *Ramaria* sp.

MATERIALS AND METHODS

L. nuda strains

All strains were deposited in the Saprobe and Mycorrhizal Fungi Strain Collection, at Departamento de Microbiología, Facultad de Ciencias Químicas y Farmacia, Universidad de San Carlos de Guatemala. The code and source of the strains are as follows: 17.01 (Tecpán-Guatemala, Chimaltenango), 54.02 (94 Km Interamerican Highway, Tecpán-Guatemala, Chimaltenango), 50.09 (San Juan Comalapa, Chimaltenango), 4.10 (San Jorge Muxbal, Santa Catarina Pinula, Guatemala) and 21.10 (Tecpán-Guatemala, Chimaltenango municipal market).

Biomass production of *L. nuda* strains

Pure cultures of different strains were prepared on malt extract agar and incubated at 26°C in the dark for 30 days. Subculturing was done on potato dextrose agar and mycelium was incubated at 26 °C for 15 to 20 days, according to the procedure recommended by Quimio & Chang (1990) and Bran *et al.* (2011).

Spawn

Spawn was prepared on wheat grains, which were soaked for 24 hours until they reached about 43% moisture and then boiled for 20 minutes. Subsequently, 200 g were placed in polypropylene bags with $CaCO_3$ (1% w/w) and sterilized (1 h, 121 °C). Five fragments of mycelium (1.0 cm^2) of each of the *L. nuda* strains were inoculated on the grains and incubated at 26°C in the dark, until growth of the mycelium was observed (Quimio & Chang, 1990; Bran *et al.* 2015).

Substrates for basidiome production

Cultivation of *L. nuda* was studied using polypropylene bags in units of 1 kg of compost that typically is used for cultivation of *Agaricus* (substrate 1, S1), wheat straw supplemented with either 25% rice bran (substrate 2, S2) or 5% soy flour (substrate 3, S3) (substrates 2 and 3 were sterilized, 2 h, 121°C) (Stott *et al.*1996, Sierra-Fernández *et al.* 2002, Bran *et al.* 2011).

Determination of moisture, dry weight and C:N ratio of the substrates

Five samples of the substrates were randomly taken. The moisture percentage was determined using a moisture analyzer MB35 (OHAUS®), then the dry weight average (g) was calculated for the substrates. The C:N ratio of the substrates was determined by Unit of Quimica de Suelos, Facultad de Agronomía, Universidad de San Carlos de Guatemala, and obtained the following results: Substrate 1 (20.6), Substrate 2 (48.2), Substrate 3 (40.8) and wheat straw (67.8).

Incubation and fructification

The procedure was based on methods proposed by Guinberteau *et al.* (1989), Stott *et al.* (1996), Sierra-Fernández *et al.* (2002) and Bran *et al.* (2012). Substrates were inoculated with 200 g of spawn of the *L. nuda* strains and incubated at 22 to 24°C until the mycelium completely colonized the substrates. Ten replicates per substrate and *L. nuda* strains were prepared. When the mycelium colonized substrate S1, it was overlaid with approximately 5 cm of sterilized and neutralized peat (Canadian Sphagnum Peat Moss®). A thermal shock (8°C) was performed to induce production of fruit bodies. Bags were then moved to a rustically crafted culture room. Environmental parameters recorded during production time were temperature (18 to 22°C) and humidity (75 to 85%). Substrates were irrigated by spraying with fresh water three times a day.

Quantification of fruit body production

Fruiting time was defined as number of days from substrate inoculation to first harvest. Fruit bodies produced were collected, weighed (g) and counted for one flush. Also, pileus diameter was measured and basidiomes were classified into group 1 (G1) <2 cm, group 2 (G2) 2-4 cm and (G3) > 4 cm. Percentage biological efficiency (BE) was determined using the formula as follows: BE = Biomass (fresh weight) x 100/Substrate weight (dry basis) (Chang & Miles 2004).

Statistical analysis

An analysis of variance (ANOVA) was performed by a SPSS®19 statistical program for BE, fruiting time and pileus diameter groups. Means were separated using Duncan's multiple comparison test with $\alpha = 0.05$ (Nurosis 2011).

RESULTS

Lepista nuda strains 17.01, 50.09 and 21.10 fruiting on substrate S1, however only strain 21.10 produced fruit bodies on substrates S2 and S3. Strain 4.10 colonized the substrates but did not produce fruit bodies and the strain 54.02 did not colonize the substrates. In general, all strains fructified in less time in substrate S1. However, strain 21.10 had the shortest fruiting time and was statistically significant with respect to the others ($p<0.05$). Strains 17.01 and 50.09 did not show statistical difference in this same substrate ($p>0.05$). The fruiting time in substrates S2 and S3 in strain 21.10 was statistically significant ($p=0.003$) and showed the longest fruiting times (Table 1, Figure 2).

Highest BE was obtained by strain 21.10 in substrate S2, which was significantly different with respect to the other strains and substrates evaluated ($p<0.05$). BEs for this same strain in substrates S1 and S3 were not significantly differently ($p = 0.508$). There was no significant difference in BEs from strains 17.01 and 50.09 on substrate S1 ($p> 0.05$). Strain 50.09 had the lowest BE in substrate S1 (Table 1).

Table 1. Biological efficiency for *L. nuda* strains cultivated on three substrates.

Strain	Substrate[1]	Fruiting time[2,3]		BE (%)[3]	
21.10	S1	121.80 ± 3.96	a[3]	32.80 ± 08.90	b[4]
	S2	186.40 ± 1.82	c	57.06 ± 26.34	a
	S3	196.60 ± 1.82	d	18.27 ± 18.08	b, c
17.01	S1	137.60 ± 2.61	b	09.01 ± 02.46	c
	S2	0		0	
	S3	0		0	
50.09	S1	137.40 ± 6.58	b	02.08 ± 00.79	c
	S2	0		0	
	S3	0		0	

[1]Compost used for cultivation of *Agaricus* (S1), wheat straw supplemented with 25% rice bran (S2), wheat straw supplemented with 5% soy flour (S3). [2]Days from substrate inoculation to first harvest. [3]Mean ± standard deviation. [4]Different letters in the same column indicate statistically significant difference according to Duncan's multiple comparison test ($\alpha = 0.05$).

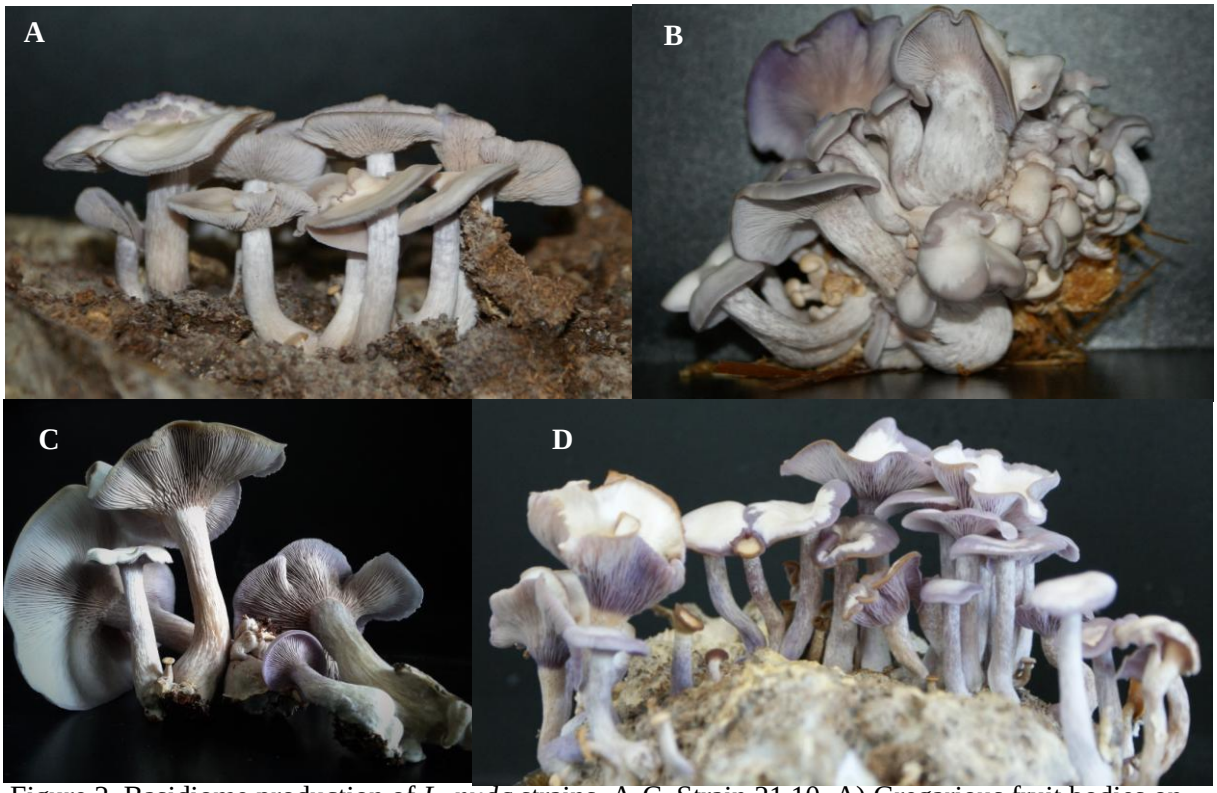

Figure 2. Basidiome production of *L. nuda* strains. A-C. Strain 21.10. A) Gregarious fruit bodies on substrate S1. B) Caespitose fruit bodies on substrate S2. C) Fruit bodies harvested from substrate S3. D) Gregarious fruit bodies on substrate S1 (strain 17.01).

The overall effect of strains on BE showed that strain 21.10 was statistically significant with respect to the others ($p<0.05$). There was no significant difference for BE for strains 17.01 and 50.09 (Figure 3). Substrate was not a significant factor for BE for substrates S1 and S3 (Figure 4).

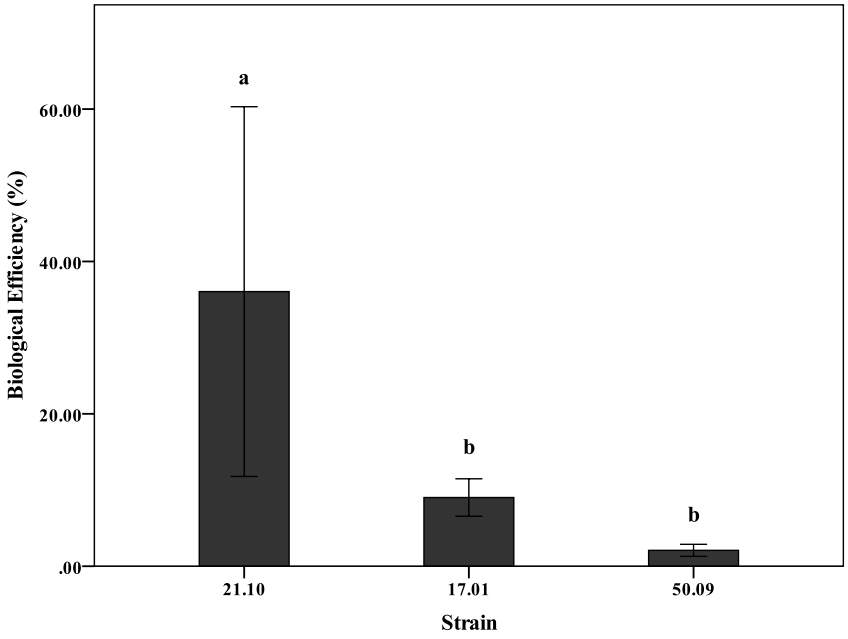

Figure 3. BE of *L. nuda* strains on evaluated substrates. Bars represent the BE mean obtained from three substrates. Error bars indicate ± 95% confidence interval. Different letters indicate statistically significant difference according to Duncan's multiple comparison test ($\alpha = 0.05$).

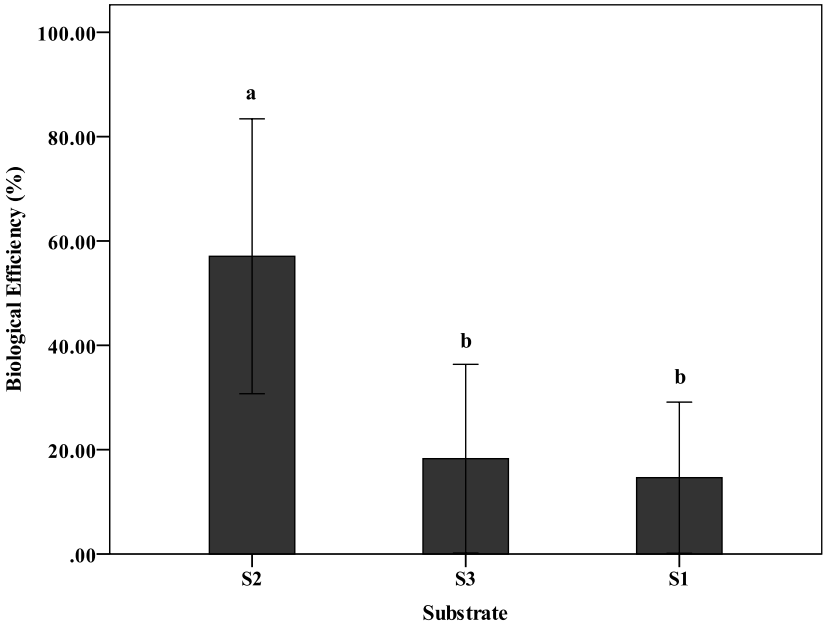

Figure 4. General effect of the substrates on the EB of the *L. nuda* strains. The bars represent the EB mean obtained in the substrates evaluated. The error bars indicate ± the standard deviation from the mean to 95% confidence interval. Different letters indicate statistically significant difference, according to Duncan's multiple comparison test ($\alpha = 0.05$).

Table 2. Pileus diameters (cm) of *L. nuda* strains produced in three substrates.

Strain	Substrate[1]	G1[2,3]	G2[2,3]	G3[2,3]	Total[3]	
21.10	S1	1.37 ± 0.23	2.90 ± 0.43	6.19 ± 0.61	3.91 ± 2.30	a[4]
	S2	1.32 ± 0.04	2.75 ± 0.19	5.47 ± 0.44	2.61 ± 1.65	b
	S3	1.31 ± 0.15	2.13 ± 1.22	3.36 ± 3.15	2.34 ± 1.48	b
17.01	S1	1.37 ± 0.15	2.37 ± 01.34	04.84 ± 0.36	2.64 ± 1.39	b
	S2	0	0	0	0	
	S3	0	0	0	0	
50.09	S1	0.73 ± 0.69	2.58 ± 1.45	4.41 ± 2.57	2.95 ± 2.08	b
	S2	0	0	0	0	
	S3	0	0	0	0	

[1]Compost used for cultivation of *Agaricus* (S1), wheat straw supplemented with 25% rice bran (S2), wheat straw supplemented with 5% soy flour (S3). [2]Pileus diameter groups: G1 (<2 cm), G2 (2 to 4 cm) and G3 (> 4 cm). [3]Mean ± standard deviation. [4]Different letters in the column indicate statistically significant difference, according to Duncan's multiple comparison test ($\alpha = 0.05$).

Strains 17.01, 50.09 and 21.10, produced fruit bodies with pileus diameter in three classification groups (G1, G2 and G3) on substrate S1. Only strain 21.10 produced fruit bodies of the three categories on substrates S2 and S3. Strain 21.10 produced the largest pileus diameter in substrate S1, which was statistically significant with respect to the others ($p<0.05$). Pileus diameters obtained by this same strain in substrates S2 and S3, as well as by the strains 17.01 and 50.09 in the substrate S1 no showed statistically significant difference ($p>0.05$) (Table 2, Figure 5).

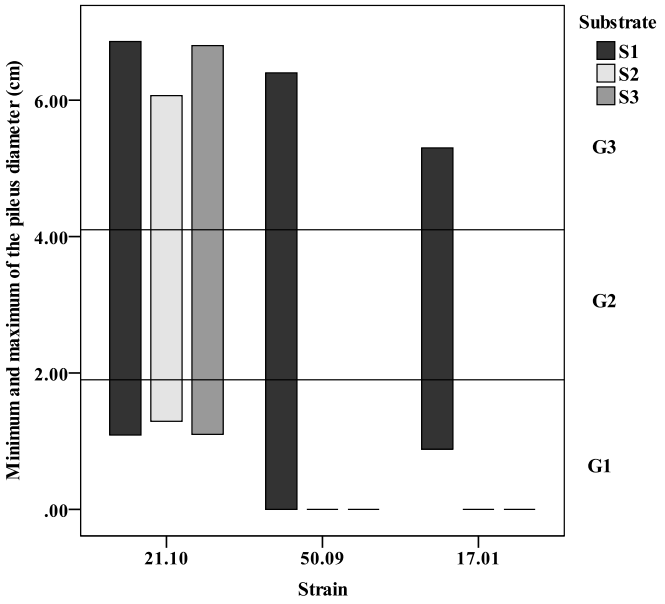

Figure 5. Classification of pileus diameter produced by *L. nuda* strains on three substrates: Compost used for cultivation of *Agaricus* (S1), wheat straw supplemented with 25% rice bran (S2), wheat straw supplemented with 5%

soy flour (S3). Bars represent ranges of minimum and maximum values of pileus diameter. Horizontal lines indicate groups of classification of pileus diameter groups: G1 (<2 cm), G2 (2 to 4 cm) and G3 (> 4 cm).

DISCUSSION

Literature indicates that cultivation of *L. nuda* is a long-time process, since that strains colonize substrates between 25 to 60 days and fruiting occurs between 24 and 52 weeks (168 to 364 days). In addition, using mycelium inoculated in beds of horse dung and straw, fruit bodies were produced after the appearance of mycelial rhizomorphs between 7 and 14 months (Stamets & Chilton 1983). Other authors report that fruiting time on compost used for cultivation of *Agaricus* lasted 7 to 10 weeks (49-70 days), producing one or two harvests with an interval of 14 to 20 days (Sierra-Fernandez *et al.* 2002).

In this study, the mycelium of *L. nuda* produced the first crop between 121-196 days in substrate S1 (compost used for cultivation of *Agaricus*). This period is longer than that reported by Sierra-Fernandez *et al.* (2002), but is within the period reported by Stamets & Chilton (1993). In the case of this substrate, fruiting time was shorter than that observed in substrates S2 and S3. This may be due to the C:N ratio of 20.6, which is very close to the optimal ratio (15-16) for development of *Agaricus* (Vedder 1996).

Fruiting time observed for *L. nuda* strain 21.10 in substrates S2 and S3, consisting of wheat straw supplemented with 25% rice bran or 5% soybean meal respectively, coincides with the period reported by Stamets & Chilton (1983). The delay in colonization by *L. nuda* strain 21.10 of substrates S2 and S3 (186-196 days) compared to substrate S1, may be due to the high initial carbon nitrogen (C:N) ratio (48.2 and 40.8, respectively). Microorganisms, in general, require a C:N ratio of 20 to 30 to initiate the degradation process (Roy *et al.* 1981). Wheat straw only substrate (C:N ratio 67.8), therefore, would need to be supplemented to increase nitrogen levels for degradation by this fungus.

Highest BE (57.06%, strain 21.10) was obtained on the substrate composed of wheat straw supplemented with 25% rice bran (S2). Strains 17.01 and 50.09 did not fructify on substrates S2 and S3 (wheat straw supplemented with 5% soybean meal or with 5% soy flour, respectively). Because use of supplemented wheat straw has not been reported for *L. nuda* cultivation, it is not possible to compare our results with others. Stott *et al.* (1996) used compost prepared for *A. bisporus* supplemented with 10% cereal straw and found that hyphal growth increased, but they were unable to obtain fruit bodies.

BE obtained from commercial mushroom compost (substrate S1) was lower than that from substrate S2, however, strains 21.10, 17.01 and 50.09 all fructified there. This is understandable, since this substrate has been recommended for the cultivation of *L. nuda* (Guinberteau *et al.* 1989, Stott *et al.* 1996, Vedder 1996, Sierra-Fernandez *et al.* 2002, Danai *et al.* 2008). BE (32.8%) obtained in this study in substrate S1 was higher compared to 5% yield (based on wet substrate) obtained by Danai *et al.* (2008). Unfortunately, in the publication of Guinberteau *et al.* (1989), it was impossible to determine BE and, in another case, it was not possible to obtain fruit bodies (Stott *et al.* 1996).

Other studies have reported that *L. nuda* strains also fructify on supplemented oak or alder sawdust, as well as willow, poplar and maple (Stamets & Chilton 1983) or olive leaves (Castro *et al.* 2014). We suggest evaluation of other substrates to determine fruiting capacity of Guatemalan *L. nuda* strains.

Another measure of productivity of *L. nuda* strains was pileus diameter. In this study, pileus diameters obtained were significantly larger in substrate S1 with strain 21.10. All pileus diameters were smaller than maximum diameters (14-15 cm) observed in nature (Bigelow & Smith 1969, Bran *et al.* 2003a).

From a commercial point of view, each species of edible mushrooms possesses some standard diameter for merchandizing, for example, around 15 cm is optimum for the cultivated mushroom *A. bisporus* (Malloch 1976). Although *L. nuda* cultivation is recent and not widespread, the ideal pileus diameter for commercialization has not been established. However, in the wild, *L. nuda* in Guatemala reaches 10.5 to 14 cm diameter (Bran *et al.* 2003a) whereas, in the United States it can measure between 4 to 15 cm (Bigelow & Smith 1969).

Finally, the fact that strain 54.02 did not colonize the substrates and strain 4.10 did not produce fruit bodies, may be because these strains did not adapt to substrates evaluated. Another possibility may be that they lost the capacity of colonization and fruiting due to multiple transfers in laboratory media as has been reported for most fungal strains (Labarére & Bois 2001).

The results of this study demonstrated that it is possible to obtain a high BE of Guatemalan *L. nuda* strain 21.10 in the substrate consisting of wheat straw supplemented with 25% rice bran. This combination of strain and substrate may have potential for commercial cultivation of this species. This substrate is already used in the production of other mushrooms such as *Pleurotus* spp. and *A. cylindracea* (Chang & Miles 2004, Bran *et al.* 2014).

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10. SHIITAKE CULTIVATION ON STRAW: AN ALTERNATIVE FOR SUBTROPICAL REGIONS

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ABSTRACT

The traditional cultivation of shiitake (*Lentinula edodes*) on oak logs has been replaced in part by cultivation in plastic bags with sterilized sawdust since this method offers a higher biological efficiency and a shorter cultivation cycle. However, the sterilization process has a high initial installation cost and consumes more energy. The feasibility of cultivating shiitake in pasteurized straw offers a very important alternative for subtropical regions since straw is generally an easily accessible substrate. Pasteurized straw (of different cereals: wheat, barley, etc.) has become, in recent years, a suitable substrate for the cultivation of shiitake since some strains adapted to this substrate have been selected. The selection of efficient strains is fundamental because a non-sterile substrate is used. An update of the cultivation of shiitake on straw and an analysis of its possibilities of adaptation in subtropical climates is presented.

INTRODUCTION

The edible mushroom *Lentinula edodes* (Berk. & Mont.) Pegler, is known as shiitake in Japan as well as xiang-gu in China (Chen 2005). It is also popularly known as oak mushroom and is one of the best-known and most studied mushrooms. Until the 1980s, the main world producer of shiitake was Japan, using the traditional technique of cultivation in logs. However, China is currently the world's leading producer of shiitake with more than 95% of the total produced, and this species has become the most cultivated worldwide with 22% of the total, about 7.48×10^6 tons, displacing the white button mushroom (*Agaricus bisporus*) that has been relegated to the fourth position with 15% of the total (Royse *et al.* 2017). The mushroom is estimated to have been cultivated for the first time in China between 1,000 and 1,100 AD on wood logs (Chang 1993). *Lentinula edodes* in China is an important part of gastronomy, art and culture, which is why there are often diverse manifestations alluding to this species (Figure 1).

According to Chang (2001), the shiitake cultivation system can be divided into 6 different stages that involve changes in the technologies used: 1) cutting method that was developed about 1,000 years ago, 2) wood log method, based on a mushroom spawn inoculation system invented by Japanese growers in 1928, 3) plastic bag method using small plastic bags with sawdust substrate, developed in Taiwan in the early 1970s, 4) brick- or pressed-cake method introduced in Shanghai in 1979, 5) synthetic log method, developed in 1986 in Fujian Province. This innovative method has allowed China to position itself as the world's leading producer of shiitake, and 6) small rush/plastic shed method that is derived from the synthetic log method, developed and adapted in Biyang County in Henan Province. The bags are much bigger and are laid on shelves of multiple layers within the shed.

Figure 1. Jade crafts showing wild shiitake specimens.

Since the decade of 1990, the traditional system of culture has been gradually replaced by a more modern method that uses plastic bags with lignocellulosic substrates (Oei 2003, Chang and Miles 2004, Chen 2005). For the cultivation of shiitake, hardwoods are traditionally used (Chang and Miles 1989, Kozak and Krawczyk 1993, Sobata and Nall 1994), but as mentioned previously, a more efficient and faster system has focused on using a substrate with enriched sawdust that is placed in plastic bags (Przybylowicz and Donoghue 1988, Chen 2005) (Figure 2). This method has potential for relatively high yields in a short time and in relatively small spaces. Different alternative substrates have also been tested that are generally abundant locally and at an affordable price (Chen 2005). In addition to China, shiitake is produced in different countries: Japan, Korea, USA, Mexico, Brazil, France, Spain, and others, but China produces more than 95% of worldwide production (Royse *et al*. 2017). In Mexico, the production of *L. edodes* has been tested on wood chips from different trees such as *Carpinus*, *Bursera*, *Alnus*, *Quercus*, *Eliocarpus* and *Jacaranda*, among others (Mata *et al*. 1990, Morales and Martínez-Carrera 1991, Morales *et al*. 1991, Curiel Pérez *et al*. 2012, Manero Colín *et al*. 2012, Martínez-Guerrero *et al*. 2012). Tests have also been conducted on agroindustrial wastes such as coffee pulp, sugarcane bagasse and vineyard residues (Mata and Gaitán-Hernández 1992, 1994, Salmones *et al*. 1999, Gaitán-Hernandez *et al*. 2006).

Figure 2. Shiitake cultivation on sawdust substrate. a: polypropylene bags with eucalyptus substrate during incubation process, b: shiitake substrate bags during the formation of the dark layer at the end of incubation, c: culture on logs in a green house.

CULTIVATION ON WHEAT STRAW

Among the most important advantages of growing edible mushrooms in plastic bags is the possibility of using very different types of substrates. However, such substrates must be rich in their content of cellulose and lignin. In tropical and subtropical regions, diversity of substrates is abundant and often easily accessible and inexpensive. Many of these substrates can be adapted relatively easily to shiitake cultivation through simple processes such as soaking and fermentation. Various substrates have shown potential for cultivation of shiitake such as coffee husk, coffee pulp, spent coffee grounds, sugarcane bagasse, corncobs, millet straw, wheat straw, tea leaves, peanut hulls, cotton seed hulls, sunflower seed hulls, dried grass powder, water hyacinth, etc. (MushWorld 2005).

Most of the agricultural by-products used for shiitake cultivation are subjected to autoclave sterilization treatment in plastic bags. This system, although very effective, is quite expensive for its implementation in tropical and subtropical regions. In order to reduce production costs, a method has been developed to use steam-pasteurized cereal straw as a substrate for shiitake cultivation (Delpech and Olivier 1991). This process requires a rigorous selection of strains, as well as production of fast growing inoculum that helps to limit growth of antagonistic molds (Mata *et al*. 1998, 2001, 2002, Savoie *et al*. 2000, Gaitán-Hernández *et al*. 2014). In Mexico, progress has been made on cultivation of shiitake on pasteurized cereal straw

(Gaitán-Hernández and Mata 2004, Gaitán-Hernández *et al*. 2006, Peralta Márquez and Frutis Molina 2010). However, this technology has not been implemented commercially. Work in Mexico on this species has not only focused on its cultivation on alternative substrates such as those already mentioned, but research has also been carried out with the objective of selecting strains that adapt to the different substrates tested. Also, research trials have been initiated with antagonistic fungi, hybridization, inoculum improvement and culture conditions to increase yields (Mata and Guzmán 1989, Savoie *et al*. 2001, Mata *et al*. 2002, Ramírez-Carrillo and Leal-Lara 2002, Mata and Savoie 2005a,b, Gaitán-Hernández *et al*. 2014).

Wheat straw preparation

Both the composition of substrate materials and heat treatments have substantial effects on substrate selectivity. A selective substrate allows growth of shiitake mycelium but inhibits growth of competitors. To prepare a substrate, wheat straw is shredded into pieces from 4 to 6 cm in length for easy handling during pasteurization and bagging. Crushing straw might be beneficial. It is soaked in water for 6 to 12 hours at room temperature, drained and then mixed with 2 to 10% (dry weight) gypsum. Several supplements containing materials that are lacking in wheat straw itself are added to provide sufficient nutrients for shiitake mycelium. As a nitrogen and oligoelements source, soybean flour added at 4 kg per ton can increase yield by 30%, but soybean flour is more likely to lower substrate selectivity (Figure 3).

Generally, substrate selectivity decreases by addition of components rich in nitrogen or oligoelements. Therefore, it is recommended to either add only a small quantity of them, or to sterilize the substrate if a large amount of nitrogen rich components are added. Other supplements improve competitiveness of shiitake against competing molds such as *Trichoderma* sp. Peat, sawdust or other lignin derivatives not only have absorbing properties but also contain phenolic compounds that most competitors cannot degrade easily. Shiitake can degrade these lignin components with oxidative enzymes, so the shiitake mycelium has almost exclusive access to these supplements (Mata *et al*. 2001, Savoie *et al*. 2000). Phenolic compounds may also contribute to the active defense of *L. edodes*. When mycelia of shiitake and *Trichoderma* are confronted on wheat straw substrate, shiitake mycelium forms a barrier with dense mycelium, initially white that then turns dark brown. In this dark zone, shiitake produces a quantity of enzymes from the group of laccases that serve to defend themselves from *Trichoderma*. (Figure 4). For instance, when 10% peat moss was added to a wheat substrate, the contamination rate by *Trichoderma* was reduced by 50% and mushroom yield increased by 30% (Mata *et al*. 1998).

Heat treatment with steam

Wheat straw must be pasteurized in order to kill possible competitor microorganisms as well as insects in the straw. Another goal of pasteurization is to propagate thermophilic microorganisms that will improve substrate selectivity by immobilizing readily available nutrients to competitors and by producing toxic or inhibitory molecules to limit rapid growth of competitors. The substrate mixture is placed in containers or directly in special rooms for pasteurization with steam at 65 ºC for 12 to 24 hours and then cooled to room temperature (Figure 5). Water content of substrates after pasteurization must be about 70% (Mata *et al*. 1998). During the pasteurization process, it is very important to encourage vapor recirculation in order that the temperature across the substrate is homogeneous. The most efficient way to perform this operation is to use motors that allow steam to move from the bottom to the top of the pasteurization room. If possible, the steam recirculation system should have an air filter to favor the cooling of the substrate at the end of the process. It is very important to maintain the temperature during the pasteurization process at 65°C. If the temperature increases too much there will be a decrease in the population of thermophilic organisms and if the substrate is spawned in non-sterile conditions it is highly susceptible to contamination.

However, if treatment of the substrate is adequate, a selective substrate will be obtained that will favor mycelial development of shiitake (Mata *et al.* 1998).

Figure 3. Preparation of wheat straw for shiitake cultivation. a: chopped, fragmented straw, b: fermentation of substrate in a covered area, c: addition of supplements for small-scale cultivation.

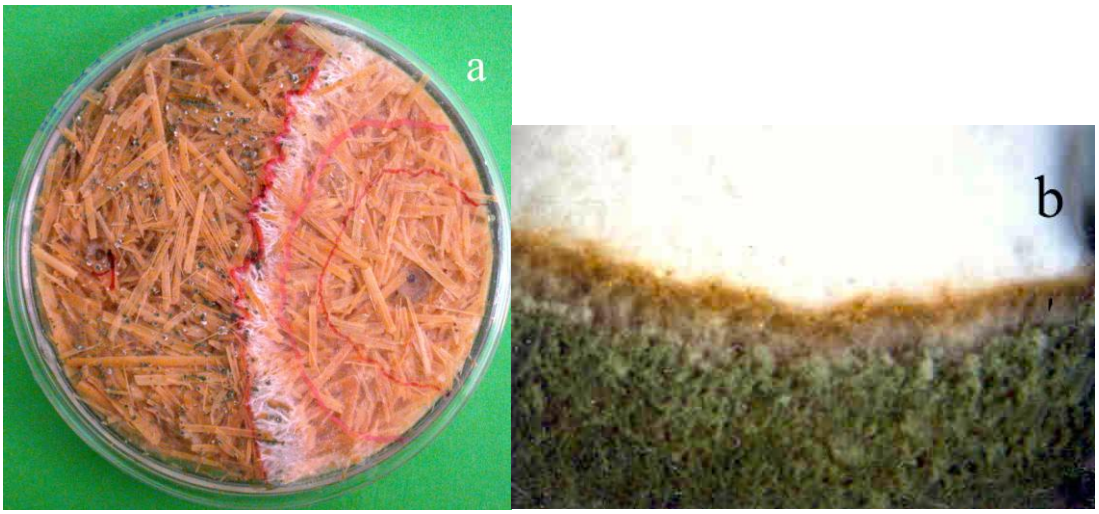

Figure 4. Confrontation of mycelia of shiitake and *Trichoderma*. a: formation of a mycelial barrier between shiitake (right) and *Trichoderma* (left), b: mycelium of shiitake forming a dark zone with high laccase production (top = mycelium of *L. edodes*, bottom = *Trichoderma harzianum*).

Figure 5. Different systems for straw pasteurization with steam. a: single-door pasteurization room for bulk substrate, b: pasteurization system type "box", c: pasteurization system of double door (outdoor-indoor), with rollers, for substrate in containers, d: single door pasteurization system for substrate in containers, e: detail of the motor for steam recirculation and air filter, f: metallic container for fermenting or pasteurizing straw.

Spawning substrate and incubation

After cooling, pasteurized substrates are mixed with spawn in a clean environment. Aseptic conditions are not necessary because the substrate is not sterilized. In order to improve competitiveness of shiitake during the first days after spawning, mycelium in spawn must be vigorous, adapted to components of the substrate and able to colonize all particles. For these reasons, Delpech and Olivier (1991) recommended limiting use of supplementation in wheat straw substrates to prevent growth of bacteria and molds. Spawn must be mixed with sterilized or pasteurized straw at 5% (w/w) and the mixture should be placed in plastic bags that are lightly perforated or equipped with a microporous filter.

Selection of genotypes appropriate for chemical and structural properties of chosen substrates and thermal treatments are critically important to ensure good production of fruiting bodies in the shortest time possible. Due to relatively few shiitake strains that are well adapted for growth on pasteurized wheat straw (Levanon *et al*. 1993, Mata and Savoie 1998, Mata *et al*. 2002), it is recommended that growers using wheat straw choose a shiitake strain with high competitiveness. However, it was demonstrated that use of supplemented spawn considerably reduces substrate contamination (Mata *et al*. 1998, Savoie *et al*. 2000). Selection of good spawn adapted to cultivation substrates, as well as to thermal treatments, is critically important to ensure a high production of mushrooms in the shortest time possible. Grains of different seeds have been used as substrate to produce shiitake spawn. Millet was tested with the addition of different supplements (Mata *et al*. 1998, Mata *et al*. 2002, Gaitán *et al*. 2014) and also to increase yield of shiitake by millet supplementation of wood chip substrate (Royse 1996). Sorghum seeds have been also tested with very good results (Salmones *et al*. 1999, Peralta Márquez and Frutis Molina 2010, Martínez-Guerrero *et al*. 2012). Preparation of nutritionally-supplemented spawn as well as preadaptation of the mycelium to final components of the culture substrate have allowed for a considerable reduction in contamination during the first growth stages (Savoie *et al*. 2000).

Incubation is one of the most important phases for shiitake cultivation on alternative substrates because of the competition between shiitake and competitor molds that occurs during the first weeks. The initial rate of substrate colonization by antagonistic fungi is an important factor of the competitive interaction. If shiitake rejects an attack by the mold at this stage, no other problem is encountered. Some strains of shiitake are able to reject mold attacks under temperature and nutritive conditions favorable to them (Badham 1991) if their mycelium has colonized enough space before contacting competitor fungi (Savoie *et al*. 1998). Incubation must be carried out at 25 ºC ± 2 with a 12-hour light and 12-hour dark cycle that is recommended at least for shiitake cultivation on wheat straw for 1–2 months depending on the strain. At the end of the incubation period, the entire surface of the substrate turns brown, indicating that mycelium is ready for fructification (Przybylowicz and Donoghue 1988, Donoghue and Dennison 1996). Generally, transparent bags are used and are placed on shelves inside the incubation rooms to promote mycelial growth. Various bag sizes are used to contain 3 to 30 kg of substrate. Sometimes shiitake growers prefer to use pre-perforated bags because if the substrate has been properly prepared there should be no contamination problems. Many producers use the same incubation rooms to induce and obtain the mushrooms but some producers prefer to have specific rooms to carry out only incubation of the inoculated bags (Figure 6).

Figure 6. Incubation of shiitake on wheat straw contained in bags. a: sample of shiitake in pre-perforated plastic bags, b: shiitake substrate contained in polypropylene bag with filter, c: bags with substrate on wooden shelves in an incubation/production room, d: bags with substrate on metal shelves in an incubation room, e: samples of shiitake (without plastic bag) hanging on a shelf at the end of the incubation period. Substrate has turned brown indicating that the mycelium is ready for fruiting.

Shiitake production on pasteurized wheat straw

After the incubation period, plastic bags are removed and substrate blocks are sprinkled with cold water. Room temperature must be adjusted to 17 to 19 ºC. A relative humidity of 90% and a cycle of 12 hours light/12 hours dark are necessary to encourage mushroom development. After obtaining the first harvest, blocks can be rehydrated to induce a second flush by soaking them in water for 12 hours (Gaitán-Hernández and Mata 2004). Although it is not a general rule, soaking substrate is a method that is frequently used since mycelial growth compacts the substrate and hinders hydration of the samples. On the other hand, the dark external layer, also known as pseudosclerotium or pellicle, protects the samples from dehydration but at the same time hinders absorption of environmental humidity and causes samples to float during soaking.

Under commercial production conditions, when large blocks of supplemented and pasteurized straw are used (10-16 kg), mushrooms may be harvested for 12 to 16 weeks and biological efficiency reaches 50 to

100%. It is difficult to make comparisons between the results of different investigations since a large number of substrates, strains and particular conditions have been studied in shiitake cultivation. Table 1 shows a non-exhaustive comparison of some substrates used in the cultivation of shiitake comparatively with wheat straw. It should be taken into account that even when the same strains or substrates are used, biological efficiency can vary with the culture conditions used, such as temperature and humidity in each experiment.

Table 1. Biological efficiency obtained for cultivation of shiitake on wheat straw, with various heat treatments and spawn types, compared with other substrates.

Substrate	Heat treatment	Spawn	Biological efficiency (%)	Reference
Wheat straw	Pst	C	15.9	Delpech and Olivier 1991
		C	59.2	Mata and Savoie 1998
		S	59.0	Mata *et al*. 1998
		S	116.0	Savoie *et al*. 2000
		S	84.2	Peralta Márquez and Frutis Molina 2010
	Phw	S	55.6	Gaitán-Hernández and Mata 2004
		S	188.3	Gaitán-Hernández *et al*. 2014
	St	C	75.2	Philippoussis *et al*. 2007
		C	66.8	Sharma *et al*. 2013
Paddy straw	St	C	50.1	Puri 2012
Coffee residues	St	C	88.6	Leifa *et al*. 1999
		C	64.3	Mata and Gaitán-Hernández 1994
		C	90.0	Fan and Soccol 2005
Sunflower seeds hulls	St	C	108.0	Curvetto *et al*. 2005
Vineyard pruning	St	C	93.3	Gaitán-Hernández *et al*. 2006
Corn cobs	St	C	80.6	Philippoussis *et al*. 2007
Sugar cane bagasse	St	S	133.4	Salmones *et al*. 1999
Sawdust	Stst	C	25.0	Thevasingh *et al*. 2005
	St	C	60.0	Fan *et al*. 2005b
		C	79.0	Sobal *et al*. 2010
		C	168.0	Curiel Pérez *et al*. 2012
		C	70.4	Manero Colín *et al*. 2012
		C	103.0	Martínez-Guerrero *et al*. 2012
	Pst	C	80.4	Royse and Sánchez 2007

Pst = pasteurization with steam, Phw = pasteurization in hot water, St = sterilization, Stst = sterilization with steam, C = conventional, S = supplemented.

Generally, the first flush of mushrooms obtained from wheat straw substrate is the most abundant with 50-70% of the total harvest, depending on strain and growing conditions (Mata *et al*. 1998, Gaitán-Hernández *et al*. 2014). Mushrooms should be harvested when they are turgid and before the pileus extends fully (Figure 7). Harvest may be done by hand, grasping the mushrooms at the base and turning them slightly so that they can be removed without physical damage. Some strains of shiitake produce mushrooms with many scales while others produce smoother fruitbodies. Through control of ventilation and humidity, final morphology of harvested mushrooms may be influenced. In several Asian countries, the "flower shiitake" presents a cracked pileus and is very appreciated. The flower pattern is not a characteristic of a particular genotype or genetically inherent trait, but rather a morphological, flower-like cracking pattern on the upper surface of the cap. This white cracking pattern is produced by different growth rates between the surface and the inner tissue of the cap due to drastic diurnal fluctuations of temperature and humidity.

Flower shiitake production requires high humidity and temperature in daytime and lower humidity and temperature during nighttime (Fan *et al*. 2005a) (Figure 8a).

Figure 7. Production of shiitake on wheat straw. a: formation of primordia, b-d: mushrooms ready to harvest.

Perhaps one of the biggest advantages of shiitake cultivation is that a major part of its market is directed to dried product. Many people prefer fresh shiitake as it combines very well when cooked with vegetables. However, dried shiitake has a particular aroma that has placed it in the preferences of consumers in the international market. Although there are different commercial presentations of shiitake that include fresh, canned, dried and canned products such as brine, many producers prefer to dry shiitake and store it and then sell larger volumes (Figure 8).

Figure 8. Commercial presentation of shiitake mushrooms. a: fresh "flower mushrooms", b: fresh mushrooms with special packaging, c: slices of dried mushrooms, d: whole dried mushrooms.

The purpose of the drying process is, in addition to dehydrating the product for its preservation, to maintain its appearance and color as well as enhancing its aroma. Drying may be done in a traditional way by exposing the product to the sun or using dryers in a chamber with recirculation of hot air. Size of the drying chamber varies depending on the production scale. The drying chamber should be maintained at 40 to 50 ºC for 24 hours. Dried shiitake should be cooled one hour before termination of the drying process and then should be placed into polyethylene bags, sealed and kept in a dry, cool and dark place. Shiitake produced by this method have better quality including better hygienic conditions and brighter color compared to sun-dried mushrooms (Fan *et al*. 2005b) (Figure 9).

Figure 9. Electric dryer for shiitake. a: metallic cabinet, b: extraction fan, c: wooden trays for placement of mushrooms.

The growing shiitake market has prompted producers to offer new shiitake-based products that are appealing to the consumer and that quickly position themselves in new niches within the market. In addition to health care products such as extracts or tisanes, one may now find a variety of snacks produced with shiitake and beers and different preserves (Figure 10).

Figure 10. Various products based on shiitake. a: shiitake and enokitake (*Flammulina velutipes*) brine, b: snacks based on shiitake, c: dry shiitake cooked and seasoned, d: shiitake beer.

Aroma characteristics of shiitake produced on wheat straw

Aroma is one of the most important characteristics of shiitake. Due to its organoleptic properties, *L. edodes* was introduced into the French market as the "fragrant mushroom" (champignon parfumé) (Olivier *et al*. 1991) where it is used in gourmet dishes. Dried shiitake possesses a fragrance that is not found in fresh mushrooms and it is related to the presence of lenthionine plus some other sulfur-based odor compounds and alcohols. In addition to lenthionine, substances such as 1,2,4-trithiolane and 1,2,4,6-tetrathiepane play an important role in the odor of dried shiitake (Hiraide *et al*. 2004, Hiraide 2006). Volatile compounds of shiitake produced in logs or in synthetic logs have been widely studied and identified as complex metabolites (Wu and Wang 2000) that may be increased by adding cysteine (or methionine), glutamic acid or other amino acids to the medium (Hiraide 2006, Hiraide *et al*. 2010).

It is important to note that volatile compounds of fresh shiitake vary depending on the substrate used for its cultivation (Mata *et al*., 2014). In this way, perhaps, specialists can differentiate a mushroom grown on an oak wood-based substrate from a substrate that has been produced on wheat straw or some other cereal. According to Mata *et al*. (2014), when shiitake was cultivated on 4 different substrates, 8 volatile compounds were obtained from the basidiocarps with differences depending on the substrates (Table 2). The most abundant compound (3-octanone) was present in shiitake cultivated on all substrates evaluated. The substrate based on oak wood showed a greater diversity in volatile compounds and the only one in which the mushrooms produced 2-pentylfuran and limonene. With this evidence it is expected that in addition to the aroma, some of the medicinal compounds that have been recognized in shiitake, would be affected when the fungus is produced on different substrates. For these reasons, it is expected that medicinal properties would also vary depending on substrate. Consequently, shiitake grown in cereal straw, does not have the same aroma and its medicinal properties could be different when it is cultivated on oak wood. However, more studies are needed to understand the effect of substrates on the production of various shiitake metabolites.

Table 2. Main volatile compounds identified in shiitake cultivated on various substrates.

Compound	Substrates				Percentage	Aroma
	1	2	3	4		
3-octanone	X	X	X	X	71.4 - 97.8	Sweet fruity
1-octen-3-ol	X	X			9.9 - 18.9	Mushroom, butter, resinous
Benzaldehyde	X			X	0.6 - 4.2	Bitter almond
Benzeneacetaldehyde	X			X	0.8 - 5.8	Fruity
2-penten-1-ol		X	X		2.2 - 9.3	Mushroom-like odor
1,2,4-trithiolane		X			1.2	Egg and garlic
2-pentylfuran				X	11.8	Butter, cooked rice
Limonene				X	6.9	Citric

Substrates: 1= Barley straw 84 % - oak powder 16 %, 2= sugarcane bagasse 100%, 3= Beech tree litter 84 % - oak powder 16 %, 4= oak sawdust 84 % - oak powder 16 %. Modified from Mata *et al*. 2014.

Some pests and diseases encountered during shiitake cultivation

Like all cultivated mushrooms, production of shiitake may be seriously affected by the development of some pests and diseases. With cultivation of shiitake on logs, more than 150 species of fungi are known competitors (Lou 1981). For outdoor shiitake crops, it is practically impossible to control spore dispersal of wood destroyers since they are transported by air. According to damage they may produce during the crop cycle, these fungi can be classified into three categories: a) disease-causing fungi, i.e., they are capable of attacking and killing shiitake mycelium, b) competitor fungi, i.e., they do not attack shiitake

mycelium but decrease the amount of nutrients available for its growth and development, and c) weed fungi, i.e., they are not usually a problem but sometimes can act as competitors (Przybylowicz and Donoghue 1988). In addition to fungi, some insects and other pests may also reduce production of shiitake on logs. Insects that might damage cultivated shiitake by affecting logs or fruiting bodies include termites, beetles (Families Cerambycidae, Scolytidae, Erotylidae), moths, flies and springtails. Slugs and snails are probably the most commonly encountered animal pests of shiitake and they cause serious damage by feeding directly on mushroom caps. Some mammals also like to eat shiitake fruit bodies (deer, mice, squirrels, rabbits and pigs) (Bak and Kwon 2005).

Fortunately, for cultivation of shiitake in bags, diseases and pests are limited when compared to those found during log cultivation. In most cases, contamination that occurs in bags with cereal straw is due to fungi of the genus *Trichoderma* (Fan *et al*. 2005c). *Trichoderma*, the asexual stage of the genus *Hypocrea* (Ascomycetes), is common in natural habitats containing organic matter (Samuels 1996). *Trichoderma harzianum* Rifai, which has a rapid mycelial growth rate, is the cause of green mold, a disorder that affects cultivated mushrooms such as *Lentinula edodes*. Shiitake resists *Trichoderma* spp. attack by producing high quantities of laccases (p-phenol oxidase, EC 1.10.3.2) in barrages that are characterized by brown line formation in contact zones of the mycelia (Savoie and Mata 1999) (Figure 11, see also Figure 4). Laccase production is also induced by extracellular metabolites produced by *T. harzianum* (Savoie *et al*. 1998). Extracellular laccase production by shiitake is a defensive metabolite produced during interaction with *T. harzianum*. This characteristic of shiitake allows stimulation of the production of some enzymes that are considered important at the beginning of mycelial growth. To stimulate production of laccase, phenolic compounds may be added to grain used in spawn preparation. This increases the probability of resisting antagonistic fungi in early stages of mycelial development during incubation (Mata *et al*. 1998). On the other hand, antagonistic relationships between shiitake and molds depend enormously on the composition and thermic treatment of the final production substrate. For this reason, it is very important to properly perform pasteurization of substrate and not exceed the recommended temperature (65 ºC) to favor development of microorganisms that inhibit the growth of *Trichoderma* (Mata *et al*. 1998). Supplementation of substrate is recommended, but when a steam pasteurization method is followed, supplementation should be light and preferably by adding elements that help inhibit growth of antagonist molds. After first harvest of mushrooms, it is necessary to check substrate blocks frequently, as *Trichoderma* spots may begin to appear over time (Figure 11).

A substantial part of the success for control of *Trichoderma* has to do with good practices carried out during cultivation of shiitake. Strict hygiene must be maintained in all facilities including personnel access control. During spawning, access to facilities should be restricted to a minimum. If, despite all precautions taken, there is contamination by *Trichoderma*, a rapid evaluation should be made to consider whether it is necessary to eliminate all contaminated samples. Contamination of substrate with bacteria may also be a major problem. In addition to affecting mycelial growth, bacteria often attack shiitake fructifications, producing deformations and greatly decreasing production. Bacterial attacks can also produce spots on the hymenium that diminish quality of the product. Samples with severe bacterial attacks should be eliminated quickly. Sometimes, deformations of basidiocarps occur due to lack of ventilation in the cultivation room, to excess moisture or to virus infection (Figure 11). During cultivation of shiitake in bags, some pests may also appear that are attracted by the odor of fructifications. The most frequent pests encountered include mites, springtails, slugs, and small rodents (Fan *et al*. 2005c). It is also common to find ladybugs. In most cases, these pests can be controlled relatively easily through specific traps.

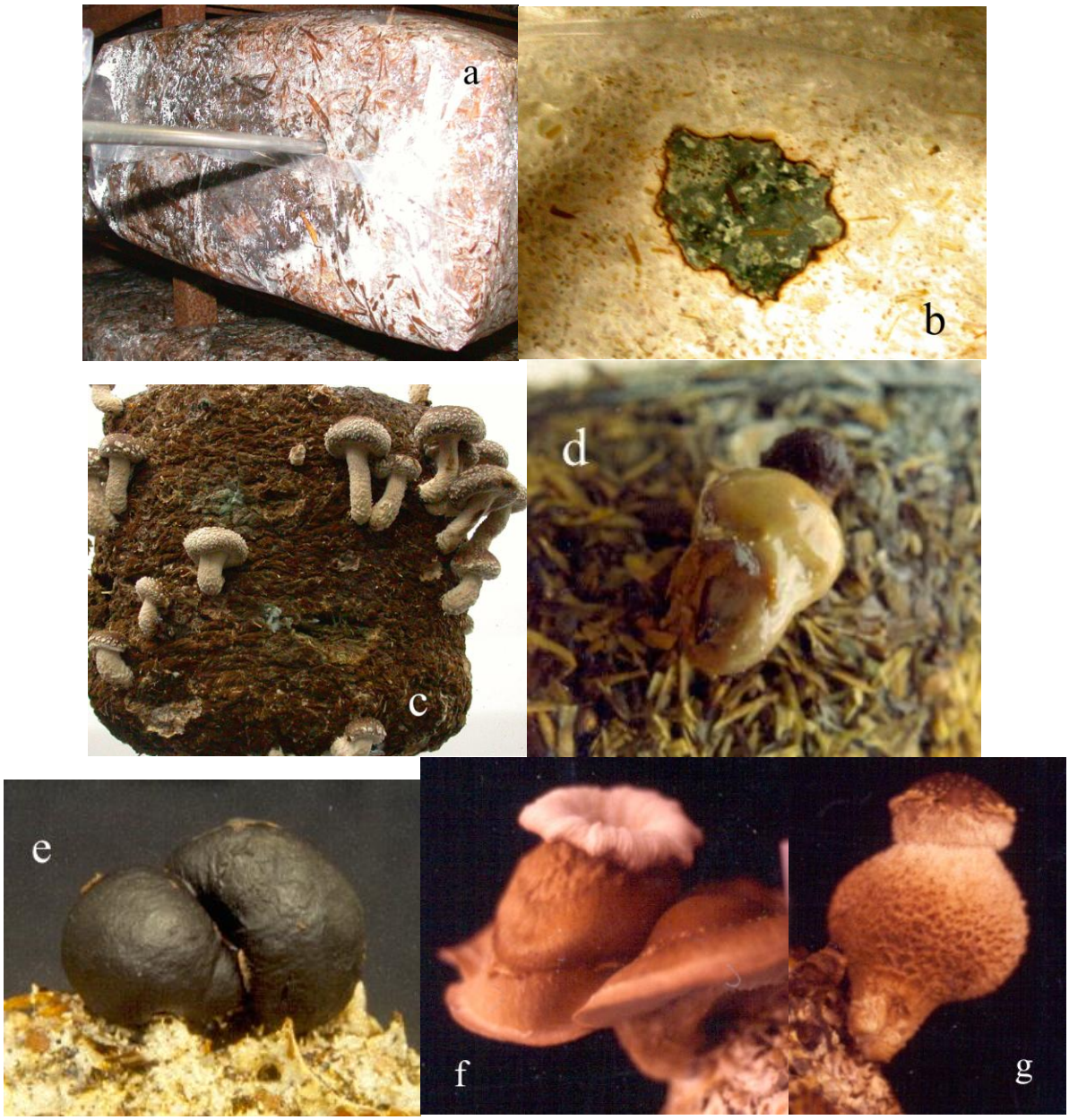

Figure 11. Contamination encountered on shiitake substrate and fruit bodies. a-b: shiitake substrate contaminated with *Trichoderma* mold, c: *Trichoderma* contaminating a shiitake block substrate during second flush, d-e: shiitake fruit bodies contaminated with bacteria, f-g: deformed shiitake fruit bodies.

THE AMERICAN SHIITAKE

Among the species that have a promising future for their cultivation is the so-called American shiitake, *Lentinula boryana* (Berk. & Mont.) Pegler (Figure 12). This species is an edible neotropical fungus that grows in subtropical and tropical forests from the southeastern United States, Mexico, the Caribbean, Central America to north of South America (Guzmán 1972, Mata and Guzmán 1991, Guzmán *et al*. 1997). Although in Mexico it is not one of the most popular species in traditional markets, it is frequently found for sale in the center of the state of Veracruz where it is known as "hongo de encino" (oak mushroom), "hongo de palo" (stick mushroom), "cuerudo" (leatherly) (Mata and Guzmán 1989) while in Chiapas it

receives the indigenous name of "nla" (Guzmán 1997). Currently there are no data on its commercial production and only a few studies have been carried out on the feasibility of its cultivation on different substrates (Mata and Guzmán 1993, Soto-Velazco *et al*. 1995, Mata *et al*. 2001, Salmones and Gutiérrez-Lecuona 2008). However, despite morphological similarities between the two species, it has been shown that these are two completely separate and genetically distinct species, they are not interfertile and although they belong to the same genus they are two phylogenetically separated species (Mata and Guzmán 1989, Guzmán *et al*. 1997, Nicholson *et al*. 1997). Besides their macroscopic similarities, both species have similar nutritional properties, suggesting the possibility of cultivating *L. boryana* using technology developed for cultivation of *L. edodes*. This would suggest that the American shiitake could be produced in large quantities. However, with this species it is necessary to carry out an intense work regarding the selection of strains.

Figure12. *Lentinula boryana*, the American shiitake, cultivated on wheat straw.

On the other hand, aroma of a species is a key feature that can help to identify and create a "personality" when trying to introduce the mushroom commercially. Differences in the aroma between *L. boryana* and *L. edodes* allow to distinguish them with relative ease (Mata *et al*. 2014). The American species, *L. boryana*, is consumed in Mexico fresh and is appreciated for its natural flavor and there is no record of any process for preservation and stimulation of the production of aroma. Despite the morphological similarity of both species, aromatic differences may provide a sufficient basis for believing that the introduction of *L. boryana* could help to diversify the supply of edible species for human consumption. The medicinal properties of *L. boryana* and its comparison with *L. edodes* remains unknown, but one might expect that the American species possesses similar properties to those of shiitake.

PERSPECTIVES

Worldwide, more than 500 species of wild mushrooms are known to possess medicinal properties, such as anti-inflammatory, antioxidant, antihypertensive, anti-cholesterol, antiviral and immune system stimulants (Pérez-Moreno *et al*. 2010). Shiitake contains β-glucans and may be beneficial for human health. It has been used for treatment of cancer, hypertension and high cholesterol levels (Bak *et al*. 2014). Edible mushrooms are currently appreciated not only for their culinary value but also for the benefits they may provide to human health. Consumers are increasingly interested and informed of the benefits they may

obtain through food and, as a result, a whole new market has been created for so-called "functional" foods. A functional food provides health benefits beyond the basic nutritional requirements, that is, it contains ingredients that have proven to help improve human health (Soler-Rivas and Reglero 2012). Nutraceuticals are products of pure fungi or partially refined, from fruiting bodies, mycelium, or from the culture medium filtered after mycelial growth in submerged culture. These products have nutritional properties that promote health and they are usually consumed in the form of capsules or tablets as a dietary supplement (Chang and Buswell 1996, Wasser *et al*. 2000, Trigos and Suárez Medellín 2010).

Edible mushroom cultivation is not only an ancestral agricultural practice, but an activity that currently requires technological and scientific knowledge. Mushroom production from agricultural wastes for both food and medicine help maintain an ecological balance that is sustainable. Edible fungi will undoubtedly, due to their health benefits, be one of the most important elements in the diet of humans in the near future.

The cultivation of shiitake in tropical and subtropical regions using straw from various cereals represents an alternative that should be considered in the near future. However, it is worth noting that research is needed to select strains with a high production capacity at temperatures close to 25 ºC. The effects of imminent global warming are becoming more and more noticeable in the temperate zones and the increase of temperature reduces favorable zones for crops of many mushroom species. Deepening knowledge of tropical and subtropical species of edible mushrooms and their management is essential for economical production of these crops. Due to its nutritional characteristics and relative ease of production, shiitake is destined to be a very important food in the near future.

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Biotechnological Applications

11. *Auricularia* spp.: EDIBLE MUSHROOMS WITH BROAD BIOTECHNOLOGICAL PROPERTIES

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ABSTRACT

An account is made of the importance, technology of cultivation and advances in medicinal and biotechnological applications of the edible species of *Auricularia*. This genus represents the third most cultivated mushroom worldwide and is the oldest cultivated by humans. It is a tropical and subtropical fungus that grows frequently in places with average temperatures between 18 and 27°C. It is grown mainly in Southeast Asia. As a formula for cultivation in different parts of the world, various sawdust and wheat straw raw materials used either alone or mixed with bran are reported, as well as supplemented corncobs. Antioxidant and medicinal qualities are mentioned in the treatment of heart problems, diabetes and certain types of cancer. From a biotechnological point of view, this genus has broad expectations of application for bioremediation of recalcitrant and emerging pollutants.

Keywords: wood ear mushroom, tropical mushrooms, medicinal uses, antioxidants

INTRODUCTION

Auricularia auricula-judae (≡*A. auricula*) is the first species of edible mushroom cultivated by humankind. There are reports on its cultivation in China, from about 300 BC using logs (Cheng and Tu 1978, Quimio 2002). Its cultivation in plastic bags is more recent, with this method developed in Taiwan in the early 1970's. *Auricularia auricula-judae* and *A. nigricans* (≡*A. polytricha*) (Chang and Quimio 1982) are the two most cultivated species, although *A. fuscosuccinea* is also cultivated to a lesser extent in China (Quimio 2002). As a whole, these species now account for around 6,264 x 10^3 t of *Auricularia* in China only, reference year 2013 (Figure 1, Chang 2005, Li 2002, Zhang *et al*. 2014.). It should be noted that cultivation of mushrooms of this genus is mainly in Southeast Asia, especially China, which produces about 90% of the total, in addition to Taiwan, Thailand, the Philippines, Indonesia and Malaysia. With current world production, the genus *Auricularia* occupies third place in production, only after *Lentinula edodes* and *Pleurotus* spp. and more than *Agaricus bisporus,* the fourth most cultivated species (Royse 2014, Zhang *et al*. 2014, Royse *et al*. 2017).

Outside of Southeast Asia, cultivation of *Auricularia* is practically nonexistent, although some cases of small-scale production have been reported in certain places in America and even Africa (Apetorgbor *et al*. 2005, Lou Hsu *Pers Comm*, Sawyer 2000, Ibáñez *et al*. 2009). The consumption of *Auricularia* occurs mainly in producing countries, although not exclusively. Thus, it is consumed in Europe and America, mainly within Asian communities and in specialized restaurants. In several regions, wild basidiomes of some species of this genus are consumed in a traditional way by the local population (Ruan-Soto *et al*. 2004, 2009, Robles *et al*. 2007).

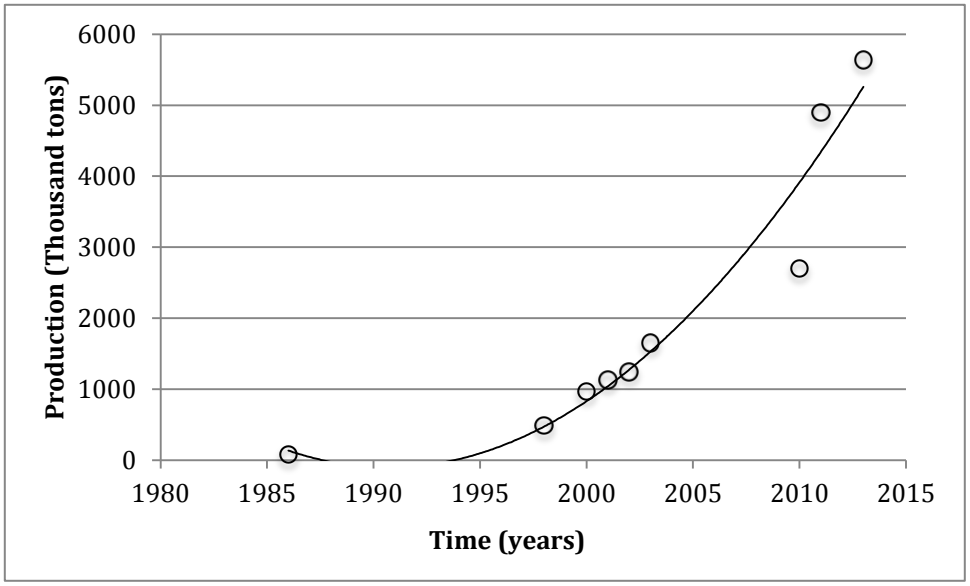


Figure 1. Production of *Auricularia* spp. in China (thousand tons). Source: Chang 2005, Li 2002, Zhang *et al*. 2014.

Substantial production and consumption of *Auricularia*, its ease of cultivation and the absence of cultivators in large regions outside of Southeast Asia, make it an excellent alternative for production and diversification. In Latin America, for example, there are growers in regions with several years' experience cultivating *Pleurotus* spp. In some of these regions, there are signs of saturation of the local market, and this likely will result in a decline in the price of oyster mushrooms with a collapse of some growers. Initiating production of an edible mushroom other than the one already grown is a good option that opens up new markets for the producer and helps maintain stability of prices.

An overview of cultivation of *Auricularia* spp., a common genus in the tropics and of growing interest, is presented here. It is detailed in some cases, with data obtained at El Colegio de la Frontera Sur (Ecosur), with species found in the state of Chiapas, Mexico and a culture of *A. fuscosuccinea.* Also, an overview of Chinese wood ear mushroom production is presented.

Auricularia spp.

Index Fungorum (2016) indicates that historically, from about 529,712 online incomes, 173 records have been named within the genus *Auricularia*. Of these, 55 are not identified to species, 43 belong to other genera and 75 names represent the 32 species so far recognized within the genus.

For the state of Chiapas, Mexico, *A. auricula-judae, A. fuscosuccinea* and *A. nigricans* (≡ *A. polytricha*) species are very frequent and grow mainly during the rainy season. These mushrooms grow on branches and stumps of plants such as coffee (*Coffea arabica*), mango (*Mangifera indica),* rubber (*Hevea brasiliensis),* primavera (*Tabebuia donnell-smithii),* laurel (*Ficus benjamina* L.), almond (*Prunus dulcis),* capulín (*P. salicifolia), Inga* spp., and *Yucca* spp, from May to December on the Mexican side of the Pacific and from June to February on the slope of the Gulf of Mexico.

Some sites within the state of Chiapas where specimens of this genus have been collected are shown in Table 1 and Figure 2. The range of sites in terms of heights above sea level and temperatures varies

widely, ranging from 100 to 150 m (Huehuetán with average temperature of 27°C and extremes minimum and maximum of 23 to 31°C) and 1800 to 1950 m (Chiquihuites, with average temperature of 18°C and a range of 13 to 23°C). Under the same conditions it is possible to find *A. delicata*, although less frequently. *Auricularia* spp. also grow on cedar trunks (*Cedrela mexicana)*, coconut (*Cocus nucifera),* cocoa (*Theobroma cacao)*, *Bursera simaruba*, chile (*Capsicum* spp.) and Mexican lemon, a variety of *Citrus limon* (Sánchez Vázquez *et al*. 1995). The four mentioned mushroom species are used for food by some settlers of diverse localities of the entity. *A. mesenterica* is another species that also grows in the region. It fructifies after the rainy season on branches and trunks and has a leathery consistency, therefore, it is not as desirable as the other species. In coffee plantations of Soconusco, it is common to find five species: *A. auricula*, *A. delicata*, *A. mesenterica*, *A. fuscosuccinea* and *A. nigricans* (≡ *polytricha*) (Andrade *et al*. 1996).

Table 1. Some municipalities where specimens of *Auricularia* spp. have been collected in Chiapas, Mexico with average, minimum and maximum temperatures during the year and height above sea level (m).

Municipality	Average temperature (°C)	Temperature range (ºC)	Altitude above sea level (m)
Cacahoatán	26	23-28	450-1700
Chiquihuites	18	13-23	1800-1950
Huehuetán	27	23-31	100-150
Salto de agua	26	20-33	200
Salvador Urbina	26	22-28	500
Tapachula	26.5	23-30	150-1200
Tila	23	18-31	1000
Tuxtla chico	26	22-29	120-450
Unión Juárez	21	17-24	800-1300
Yajalón	22	19-28	800-1100

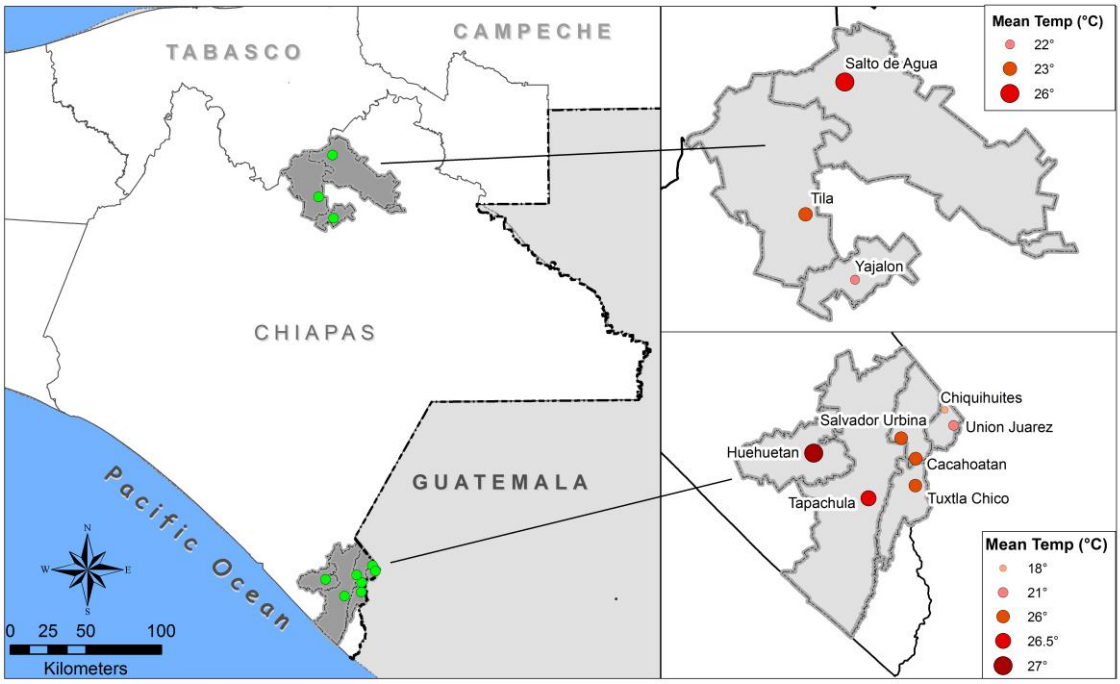


Figure 2. Areas where specimens of *Auricularia* spp. were collected in Chiapas, México.

In the Mycological Collection ECO-TA-HO- of ECOSUR, there are specimens of the five species mentioned above (Andrade and Pérez 2013). Table 2 presents a synopsis of the specimens in said collection and Table 3 shows the distribution of the specimens collected throughout the year in those municipalities.

Table 2. Specimens of *Auricularia* of the state of Chiapas present in the mycological collection of Ecosur ECO-TA HO and its frequency.

	Number of specimens	Municipalities	Localities
A auricula	45	9	30
A. delicata	80	6	28
A. fuscosuccinea	176	12	77
A. mesenterica	59	11	35
A. nigricans	222	14	76

Table 3. Species of *Auricularia* found during the year in municipalities of the state of Chiapas, Mexico.*

Municipality	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Acapetahua						M						
Cacahoatán				N	F	A-D-F-N	A-D-F-N	A-D-N	A-F-N	F-N-M	A-N	
Heuhuetán						A	N-M	N		M		
Mazatán								F	F-N		N-M	
Motozintla						F-N		N	F-N	A-F		

Ocozocuautla	N	N				A-N	M					
Salto de Agua	F									F-N	N	F-M
Suchiate							F			N-M		
Tapachula		M	N-M		D-F-N-M	D-F-N-M	D-F-N	A-D-F-N-M	A-D-F-N-M	D-F-N-M	A-D-F-N-M	N-M
Tila			F-N				D					
Tumbalá							A-F-N					
Tuxtla Chico						N	A-F-N	F-N	A-D-F-N-M	F-M	N-M	M
Tuzantán						F-N	M	N-M	F-N-M		N-M	
Unión Juárez		N			F-N	A-D-F-N	A-D-F-N	A-D-F-N	A-D-F-N	D-F-N	A-F-N	
Yajalón	F-N-M						D-F-N-M					F-N-M

*Species of *Auricularia*: A=*auricula* D= *delicata* F= *fuscosuccinea* N= *nigricans* M= *mesenterica*

CULTIVATION OF *Auricularia* spp.

Cultivation methods of *Auricularia* spp. is much like that used for other edible white rot mushrooms, such as *Pleurotus* spp. and *Lentinula edodes.* Practically, the three most popular species of this genus (*A. delicata, A. polytricha* and *A. fuscosuccinea*) can be cultivated with the same culture method, under similar environmental conditions (Wong 1993, 2016). Oei (2005), however, indicates that *A. polytricha* is the most appropriate species for cultivation in tropical regions where temperatures are usually higher and *A. auricula-judae* can only be grown in cooler areas.

The most favorable conditions reported for mycelial growth of *A. polytricha* include mycelial growth on malt extract agar, a temperature of 25-30°C and pH of 7 (Khan *et al*. 1991). In the Philippines, cultivation of *Auricularia* spp. is on a composted (five days) mixture of sawdust (78%), rice bran (20%), sugar (1%) and calcium carbonate (1%) with 65% moisture (Vilela and Silverio 1982, Oei 2005). This mixture is autoclaved for 1.5 h and then cooled for spawning. Also reported is the cultivation of *A. polytricha* on wheat straw only and supplemented with rice bran. In the latter case, a biological efficiency of 87.7 g mushrooms/100 dry substrate was obtained (Bhandal and Mehta 1989, Sharma and Jandaik 1992, Bisko and Bilay 1995). *Auricularia polytricha* was successfully cultivated on sawdust of *Acacia arabica* mixed with cotton waste (Khan *et al.* 1991, Irawati *et al.* 2012). Three types of tropical hardwoods (*Falcataria moluccana, Shorea* sp. and *Tectona grandis*) were used for the cultivation of this species with the first species giving higher yield. Information on the cultivation of *A. auricula* on sterile mixtures of sawdust with wheat bran is also available on the internet. Some of these websites are listed at the end of this chapter.

In Mexico, the cultivation of *A. fuscosuccinea* at a pilot plant level was accomplished on a mixture of sterile corncobs supplemented with *Leucaena* or tamarind leaves (Calvo Bado *et al*. 1995, 1996, Castillejos Puón *et al.* 1996). Morales *et al.* (2000) showed that the diameter of the substrate directly influences the production of basidiomata and production is better in substrate blocks with a diameter of 6-

9 cm (Figure 3). Recently it was possible to cultivate this species using a self-heating pasteurization method for substrate containing corncobs and on blends of corncobs and sawdust (Morales and Sánchez 2017.) A BE of 22.2% was obtained, which is considered low. Some research is under way using supplements to see if yield may be improved. We were able to cultivate this mushroom on sterile Pangola grass *Digitaria decumbens*, but not on this grass pasteurized by self-heating.

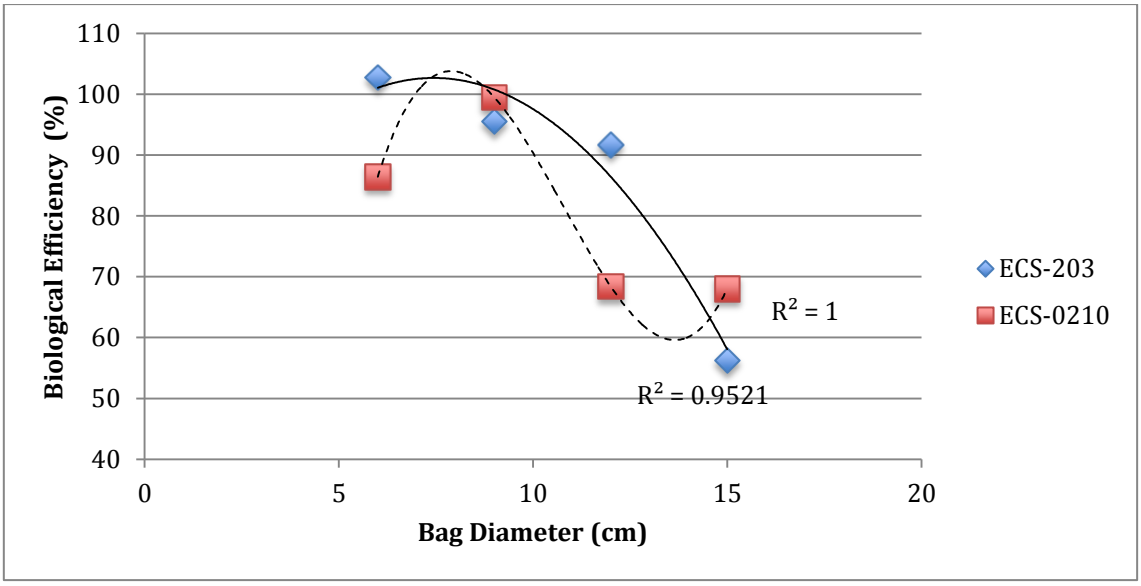
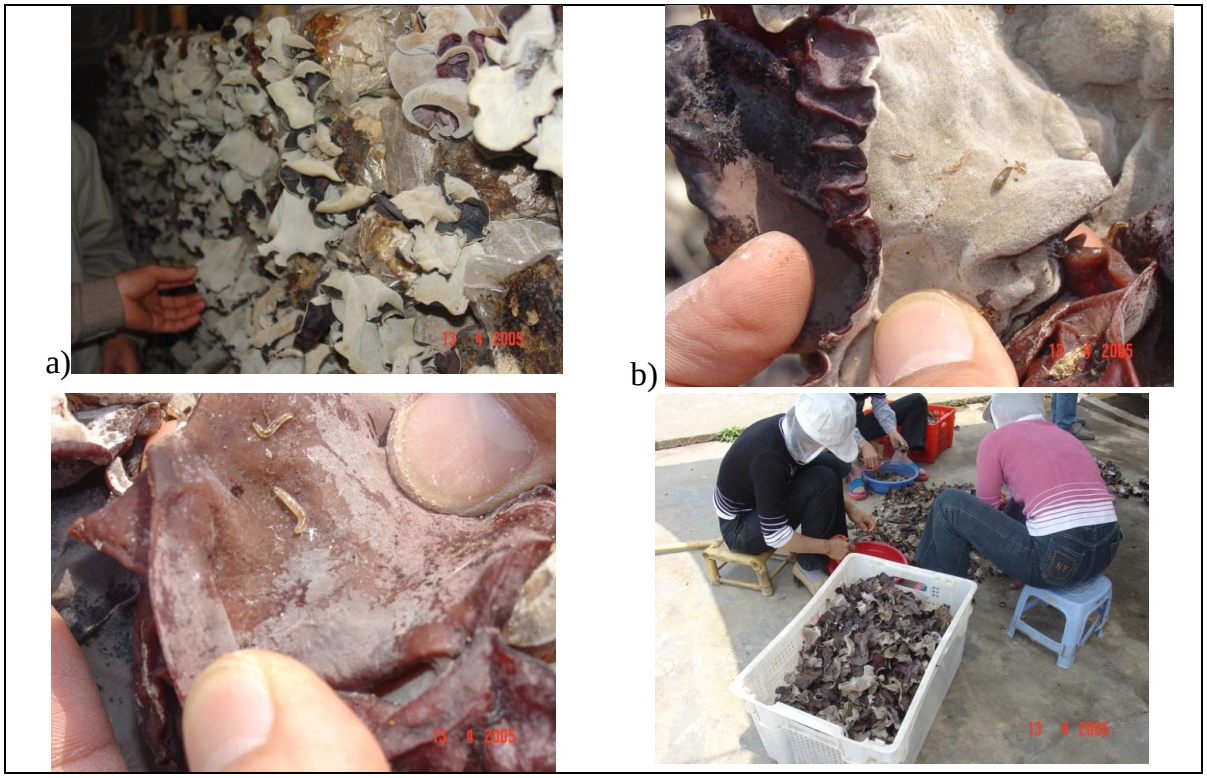


Figure 3. Effect of diameter of the substrate (corncobs) on production of basidiomes of two strains of *A. fuscosuccinea*. Source: Morales *et al.* (2000).

Figures 4 and 5 show some aspects of the cultivation of *Auricularia* spp. in China. This includes appearance of basidiomes, presence of insects and post-harvest management. In general, species of *Auricularia* are not marketed fresh, but dried. Drying is done in the sun (Figure 5d), although it is more advisable to do it in the shade (Oei 2005). Figure 6 shows some aspects of the cultivation process of *A. fuscosuccinea* at a laboratory scale in Chiapas, Mexico.

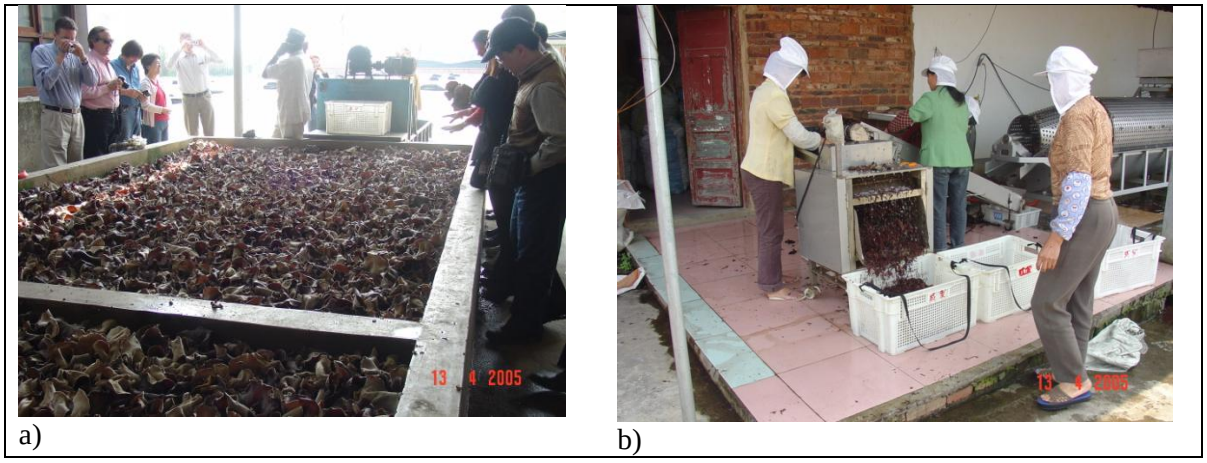
Chemical composition of *A. fuscosuccinea*

Chemical composition (as determined in the Laboratory of Bromatology of Ecosur) of *A. fuscosuccinea* cultivated on corncobs is shown in Tables 4 and 5. Protein content (13.5%) is lower than that of other cultivated mushrooms, such as the button mushroom (29-45%), or the oyster mushroom (17-25%) and it is rich in crude fiber (5.8%) relative to the button mushroom (1.9%). Carbohydrate content (62.4%) is high compared to the range observed for other edible mushrooms (38 to 70%). Also, potassium content is relatively high (Lelley 2017).



a) b) c) d)

Figure 4. Various aspects of the cultivation process of *Auricularia* spp. in China a) basidiomes of *A. polytricha* before harvest, b and c) presence of larvae in developing basidiomes, and d) quality control of harvested mushrooms.



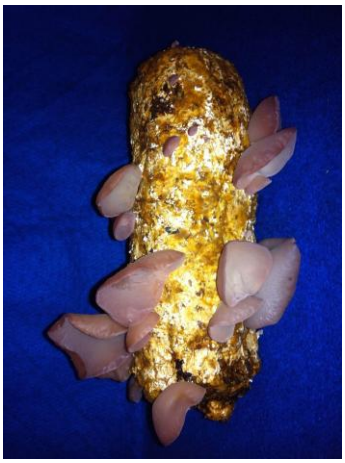
a) b)

c)

d)

Figure 5. Processing *Auricularia* spp. fruitbodies in China: a) disinfection treatment of harvested basidiomes, b) postharvest treatment chain: draining and slicing of basidiomes, c) sliced *A. auricula* basidiomes, and d) sun drying of sliced basidiomes.

a)



b)

c)

Figure 6. Basidiomes of *A. fuscosuccinea* cultivated on corncobs in Chiapas Mexico a) on the substrate (corncob), b) freshly harvested, c) dry. Photographies, a & b) Kary G. Trujillo c) René H. Andrade.

Table 4. Proximal analysis of *A. fuscosuccinea* cultivated on corncob (g/100g).

Moisture	Crude Protein	Crude Fiber	Fat	Carbohydrates	Reducing sugars	Ash	Energetic value (Kjoule)
7.9	13.5	5.8	13.5	62.4	10.3	4.7	1780.5

1 Kjoule=0.239 kcal.

Source: Bromatology Laboratory. Ecosur

Table 5. Mineral content of *Auricularia fuscosuccinea* cultivated on corncobs.

Calcium (g/100 g)	Magnesium (g/100 g)	Potassium (g/100 g)	Sodium (g/100 g)	Iron (g/100 g)	Copper (mg/kg)	Zinc (mg/kg)	Manganese (mg/kg)
0.072	0.110	1.108	0.005	0.005	Less than 0.005g/100g		

Source: Bromatology Laboratory, ECOSUR

MEDICINAL USES

Various medicinal properties are attributed to this genus, including antibiotic, anti-inflammatory, anti-diabetic, etc. Tang *et al.* (2010) reported that *A. auricula* and *A. polytricha* are used in China and other parts of Asia to treat various health conditions, such as hemorrhoids, hemoptysis, angina, diarrhea, and gastrointestinal ailments. More recently, several species of this genus are used to prevent clotting, stroke, heart attack, and to lower cholesterol and triglycerides. They also have antioxidant and useful properties for treatment of diabetes and certain types of cancer. Polysaccharides of *Auricularia auricula-judae* are known to provide myocardial protection benefits by improving superoxide dismutase (SOD) activity and reducing lipid peroxidation in the heart (Ye *et al*. 2010). Other authors have examined the ability of ethanolic extracts and polysaccharides of species of this genus for activity as antioxidants, antibiotics, hypoglycemics, hypolipidemics or hepatoprotectives (Yuan *et al*. 1998, Mau *et al*. 2001, Yang *et al.* 2011, Zeng *et al*. 2012, Cai *et al.* 2015, Reza *et al.* 2015, Fang *et al.* 2016). Antioxidant, hypoglycemic activities and anti-lipid accumulation in the liver have also been found (Yang *et al.* 2002, Sun *et al.* 2010, Chiu *et al.* 2014).

BIOTECHNOLOGICAL USES

Auricularia fuscosuccinea possesses an important ligninolytic capacity due to the presence of the enzymes laccase and phenol oxidase (Figure 7, Montoya *et al.* 2014, Yanez-Montalvo *et al.* 2015, 2016). These ligninolytic properties appear responsible for important capacities for recalcitrant substances degradation, such as the insecticide endosulfan and the growth on agroindustrial polluted effluents (Nieto López y Sánchez Vázquez 1997). In fact, Escobar *et al.* (2002) demonstrated that this species is able to degrade 100 mg/kg endosulfan in eight days of liquid culture. On the other hand, Yánez Montalvo *et al.* (2016) demonstrated that the crude extract from a liquid culture of *A. fuscosuccinea* is able to degrade 97% of this insecticide in a solution of 35 ppm in 4 days of contact, suggesting the presence and action of extracellular enzymes. Furthermore, this species is able to decolorize the bright blue R dye R Remazol (RBBR, Machado *et al.* 2005) and recent studies have demonstrated the ability of this species to degrade 100% of the emerging metamizole sodium pollutant in just six hours of exposure to a culture extract of this fungus (Mayorga *et al.* 2017).

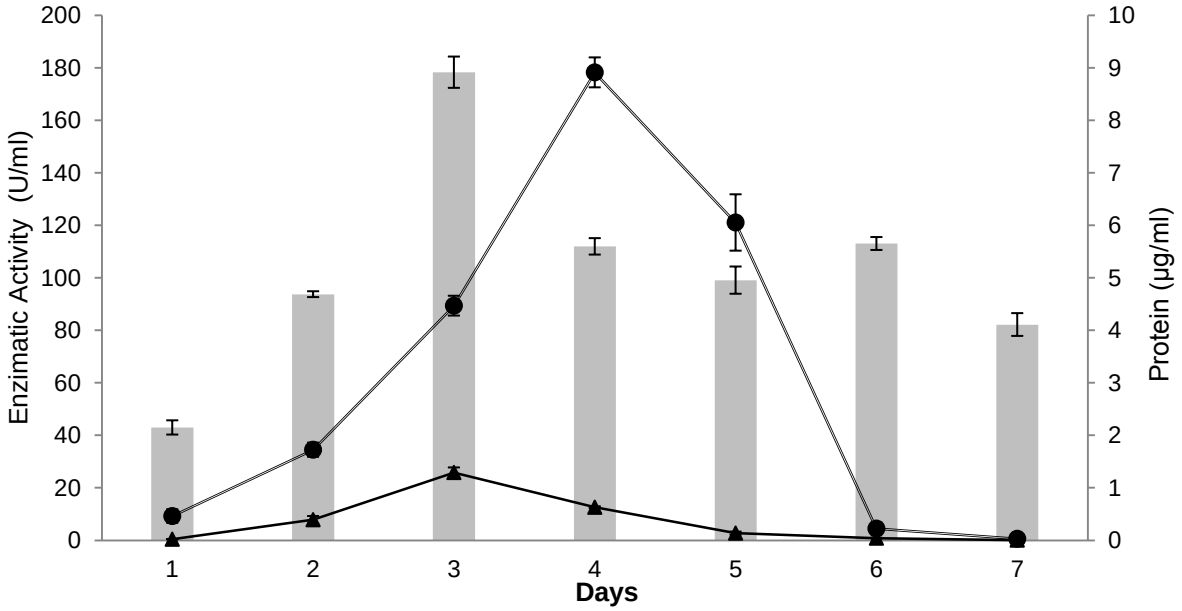


Figure 7. Production of laccase (▲), phenol oxidase (●) and protein concentration of cell free supernatant (gray bars) of liquid culture of *A. fuscosuccinea* ECS-0210, in flasks (per L) of culture medium (2 g glucose, 5 g yeast extract), during 7 days at 110 rpm and 26-28°C. Values are means of three replicates with standard deviation (Yánez-Montalvo 2014 and Yánez-Montalvo *et al.* 2015).

PERSPECTIVES

Although the first edible mushroom cultivated worldwide, about 2300 years ago, belongs to the genus *Auricularia*, this genus is not one of the most studied or known today. Other mushrooms, that were grown at a later date, but that now have significant production worldwide, such as *A. bisporus, L. edodes* and *P. ostreatus* have been studied in more detail. It is well known, for example, that the basidiomes of *Auricularia* spp. are very sensitive to and affected by insect attack, mainly of the larval state. However, there are no reports or studies carried out on this subject in cultivation. With other cultivated mushrooms, there is much more research on pest management. However, with *Auricularia* spp., much work remains in order to provide more knowledge to better control this fundamental aspect of the crop and reduce current costs in post-harvest treatments.

Information on medical and biotechnological qualities of *Auricularia* spp. is rudimentary. The important findings of the nature of its polysaccharides and their enzymes suggest that there is still much potential in this genus of edible fungus.

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12. ANTIOXIDANT ACTIVITY AND CHEMICAL COMPOSITION OF *Grifola frondosa*

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ABSTRACT

Grifola frondosa is marketed in China, Japan and other Asian countries as a medicinal and edible fungus. Several bioactive properties have been reported. This mushroom also has considerable levels of protein and its content may vary depending on culture conditions and the type of substrate used. In this study, we worked with dry and crushed fruit bodies of *Grifola frondosa* (HEMIM-43) cultivated on supplemented oak sawdust. Its proximal chemical composition, polyphenol content and inhibitory concentration (IC_{50}) of an aqueous extract with ABTS and DPPH radicals were determined. Fruit bodies contained 28.9% protein, 2.4% ash, 6.8% ethereal extract, 44.8% nitrogen free extract and 17% fiber. The content of polyphenols was 0.26 mg GAE/g and antioxidant activity of ABTS and DPPH radicals was positively correlated with extract concentration. It is possible that fruit bodies contained substances that act as donors of hydrogen and react with free radicals to turn them into stable products and to complete the chain reaction.

Keywords: *Grifola frondosa*, chemical composition, polyphenols, radical DPPH, radical ABTS.

INTRODUCTION

Grifola frondosa (Dickson: Fries), also called maitake and hen of the woods (Stamets 1993), often develops as a heavy mass (groups can weigh many kilos) on the base of stumps and on roots of oaks, elms, persimmons and other trees. Like many other fungi, optimal maitake growth conditions exist within a limited range of temperature, humidity and other environmental factors. Maitake can be found in temperate forests of northern Asia, Europe and eastern North America (Mayell 2001). An increase in forage crop areas and civilian development have combined to limit availability of maitake in the wild.

Cultivation of maitake is of recent development, only in the last three decades, so producers have been able to pass from the dependence on wild maitake to that of cultivated maitake. Maitake is often grown on substrate formulated with a combination of sawdust/bran/soybeans (in a proportion 80:10:10) (Mizuno *et* and Zhuang 1995) contained in bottles or bags. Japanese commercial cultivation, mainly for food, began in 1981 with 325 tons (Takama *et al*. 1981), and grew to 1,500 tons in 1985, 8,000 tons in 1991 and almost 10,000 tons in 1993. Commercial production of maitake worldwide is now more than 40,000 tons (Mayell 2001). Rendón-Ramírez *et al*. (2012) reported the production of *G. frondosa* on a mix of maize straw and maize oat, where they obtained fruit bodies with typical characteristics of the species and a biological efficiency of 19.9% at 120 days of incubation. In the last two decades, maitake was also grown for use as a dietary supplement. It may be the most versatile and promising medicinal supplement, although currently less well known than shiitake (*Lentinula edodes*) and reishi (*Ganoderma lucidum*). The nutritional content of mushrooms is considered to be of high quality, not only for their proteins, but also because they contain biologically active compounds considered secondary metabolites that play an important role in the structure and survival of the fungus. There are several reports that edible fungi produce a large number of these compounds, including phenolic compounds that offer a health benefit and

provide protection against chronic degenerative diseases. Therefore, these fungi are traditionally considered a fundamental part of healthy diets and additionally have been processed for nutraceuticals (Chang and Miles 2004).

Diseases caused by poor eating habits, excessive consumption of alcohol and smoking may shorten the human life span due to oxidative stress (Sohal 2002). Although reactive oxygen and nitrogen species play an important role in the maintenance of health, their excess leads to situations of oxidative stress that are detrimental (Alves *et al*. 2010). The fungi have components that influence antioxidant activity and include phenolic compounds, tocopherols, ascorbic acid, polysaccharides, terpenoids and polysaccharide-protein complexes (Kosanić *et al*. 2012). Recently, several bioactive properties of this fungus have been explored, attracting considerable attention around the world. Fruit bodies and mycelium grown in liquid culture contain antitumor polysaccharides that have been identified as glucans (β-1,6 and β-1,3) (Masuda *et al*. 2009). Research efforts have focused mainly on therapeutic effects and methods of cultivation of this edible fungus, so there is relatively less information available on its antioxidant properties. Therefore, the aim of this work was to determine the antioxidant activity of aqueous extracts of fruit bodies of *G. frondosa.*

MATERIALS AND METHODS

Drying of material

Fruit bodies of *G. frondosa* were dried immediately after harvest. Drying was performed via an airstream at 20 to 24 °C in darkness to a constant weight. They were then macerated, sieved and stored in the dark until used (Salas *et al*. 2003).

Chemical analysis

Official methods of Association of Official Analytical Chemists (AOAC) were used for determination of moisture, crude protein, ash, ethereal extract, nitrogen-free extract and fiber (AOAC 1990).

Preparation of aqueous extract

Aqueous extract of fruit bodies was prepared with 0.5 g of sample in 10 mL of water with heat treatment (at 95 °C for 5 min). Subsequently, the supernatant was recovered by centrifugation (8000 x g/15 min).

Determination of total polyphenols

Total polyphenols were measured according to the method of Singleton *et al*. (1999). The sample (0.5 ml) was added to 4.5 ml of distilled water and mixed with 0.2 ml of Folin-Ciocalteu reagent, 0.5 ml of saturated Na_2CO_3 solution and 4.3 mL of distilled water. The reaction mixtures were incubated for 60 min in the dark at room temperature. Absorbances were measured at 725 nm. Total phenolic content was expressed in mg gallic acid equivalents per g of dry sample (mg GAE/g).

Evaluation of antioxidant activity

Scavenging activity of DPPH radical

DPPH radical scavenging activity was determined according to Moraes-de-Souza *et al*. (2008) with some modifications. The reaction mixture consisted of 0.5 ml of extract, 3 ml of methanol, and 0.3 ml of 0.5 mM DPPH radical solution in methanol. After incubation for 45 min, absorbance was determined in a

spectrophotometer at 517 nm. The antioxidant activity (% inhibition) was calculated by Eq. 1,

$$\% \text{ Inhibition} = [(A\text{control} - A\text{sample})/A\text{control}] \times 100$$

Where Acontrol is the absorbance of the negative control at the moment of solution preparation and Asample is the absorbance of the sample after 45 min.

Scavenging activity of ABTS radical

ABTS radical scavenging activity was determined according to Re *et al*. (1999) with some modifications. ABTS was dissolved in water to a 7 mM concentration. The ABTS radical cation (ABTS•+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubating the mixture in the dark at room temperature for 12 to 16 hours before use. The ABTS•+ solution was diluted with water to an absorbance of 0.70 (± 0.02) at 734 nm. The reaction mixture consisted of 0.07 ml of extract and 3 ml of the ABTS radical. After incubation for 6 min, absorbance was determined in a spectrophotometer at 734 nm. The antioxidant activity was calculated by Eq. 1, where Acontrol is absorbance of the negative control at the moment of solution preparation and Asample is absorbance of the sample after 6 min.

The IC_{50} values were calculated from the graph that represents the concentration of the sample required to scavenge 50% of the ABTS or DPPH free radicals. The IC_{50} is often used to express the amount or concentration of extracts needed to scavenge 50% of the free radicals. ABTS and DPPH were expressed as mg GAE/L.

RESULTS AND DISCUSSION

Dry extract of fruiting bodies from *G. frondosa* contained 28.9% protein, 44.9% nitrogen free extract, 6.8% ethereal extract and 17% fiber (Table 1).

Table 1. Chemical composition of fruit bodies of *Grifola frondosa*

Component	Wet basis g/100g	Dry basis g/100g
Moisture	3.35 ± 0.15	0
Ash	2.29 ± 0.51	2.37
Crude protein	27.90 ± 0.20	28.87
Nitrogen-free extract (Carbohydrates)	43.38 ± 1.87	44.88
Ethereal extract (Fats)	6.55 ± 1.15	6.78
Fiber*	16.53*	17.10

* Obtained by weight difference.

In general, in terms of the amount of crude protein, the fungi are in a lower range than animal meat, but far above most other foods (Chang and Wasser 2012). In ancient times, edible wild mushrooms were popularly known in Central Europe as the "meat of the poor" because of the quality and quantity of proteins and amino acids they contain (Kalac 2013). The protein content of the fungus is considered high, ranging from 10 to 44% of dry weight (Longvah and Deosthale 1998). Petrovska (2001) analyzed the protein content of 47 species, obtaining an average of 22.8%. Uzun *et al*. (2009) studied 30 species of

fungi and obtained average protein values of 24.9%. Some authors have detected protein values of 54% and 59% in *Cantharellus cibarius* and *Lepista nuda*, respectively (Barros *et al*. 2008).

The ethereal extract content of fungi is relatively low, with values reported between 1% and 8% on a dry basis, although it may vary between species or between strains of the same species depending on several factors (Barros *et al*. 2008). Some species of the genus *Cordyceps* contain 10.06% fat, while others such as *Lentinula edodes*, *Tremella fuciformis*, *Clitocybe maxima*, *Trametes versicolor*, *Auricularia mesenterica* and *Auricularia polytricha* are below 1.5% (Ulziijargal and Mau 2011). The ratio of unsaturated to saturated fatty acids in fungi may range from 4.3 to 12.7 in wild fungi (Liu *et al*. 2012) and 0.7 to 4.5 in cultured fungi (Zhang and Ran 2005). Oleic acid and linoleic acid are the most prevalent, constituting two-thirds of all fatty acids that have been identified in fungi, followed by saturated palmitic acid (Kalac 2009).

Carbohydrates (simple sugars as monosaccharides, oligosaccharides and construction polysaccharides as glucans) vary depending on species and they constitute approximately half of the dry matter with their average values between 35 and 70 g/100 g on dry basis. The major sugars in fungi are mannitol and trehalose with mean values of 28.9 g/kg and 39.2 g/kg (dry wt basis), respectively (Kalac 2009). Fiber is a complex mixture of carbohydrates such as: chitin, mannans and glucans with proteins, waxes, saponins or phytosterols and are not digestible by humans. Edible fungi represent an important source of fiber: 4.5 to 54.5 g/100 g of dry matter (Manzi *et al*. 2004) with mean values of 40 to 90 g/kg for soluble fiber and 220 to 300 g/kg for insoluble fiber (Kalac 2009).

The polyphenol content of *Grifola frondosa* extract was 10.4± 0.001 mg GAE/ml. Polyphenols are organic compounds whose basic structure is characterized by containing at least one aromatic ring with one or more linked hydroxyl groups. These can range from simple molecules (phenolic acids, flavonoids or phenylpropanoids) to highly polymerized compounds (tannins, lignins or melanins). The phenolic content of fungi is determined by the presence, but not exclusive, of phenolic acids, as other phenolic compounds are also present depending on the species analyzed. Arbaayah and Kalsom (2013) found that phenolic content differed between species, indicating that each species has its own pathway in the metabolism of phenolic compounds. Exposure of living cells to various sources of radicals such as sunlight and chemicals may cause organisms to develop their protective systems with both enzymatic and non-enzymatic reactions. Most of the antioxidant properties present in fungi are mainly in the form of phenolic acids and flavonoids, followed by tocopherols, ascorbic acid and carotenoids (Ferreira *et al.* 2009). It is suggested that production of phenolic compounds in fungi provides defensive mechanisms for free radicals and reactive species of certain chemicals. In a study of *Pleurotus eryngii* and *P. ostreatus*, total phenol content was 0.03 mg/g and 0.09 mg/g dry weight (Kim *et al.* 2008), respectively, while in another *P. ostreatus* strain total phenol content was 0.71 mg/g dry weight (Jayakumar *et al.* 2009). Mau *et al*. (2004) obtained 1.59 mg/g phenols using the Folin-Ciocalteu reagent method from the mycelium of *G. frondosa* and Smith *et al.* (2015) reported 2.31 mg GAE/g. In both studies they established phenols as the main antioxidant component found in the methanolic extracts of the mycelium. Flavonoids represent the lowest levels of phenolic compounds found in fungi (Barros *et al*. 2008). In fact, there are few sources that prove their presence in scarce, and very specific, species (Iwashina 2000, Ribeiro *et al*. 2007).

The percentage of inhibition of both radicals increased in relation to concentration, i.e., the IC_{50} of the aqueous extract with the DDPH radical was 17.1 mg GAE/L and with ABTS was 101.3 mg GAE/L. Yeh *et al*. (2011) reported a higher percentage of DPPH radical in water extracts than in ethanolic extracts of *G. frondosa*. Percentages of inhibition were in descending order of cold water (62.6%-59.6%)> hot water (53.1%-50.8%)> ethanol (17.1%-18.6%). Inhibition of both aqueous extracts and ethanolic extract may be due to the presence of soluble polysaccharides. Percentage of inhibition of aqueous extracts was higher in cold water compared to hot water. Another study also found that the percentage inhibition of cold-water

extract was higher than that of warm-water extract of *Pleurotus citrinopileatus* fruit bodies (Lee *et al*. 2007).

Fungi with these type compounds are already available in commercial formulas with proven antioxidant activity, as in the case of powdered preparations of two of the most famous and studied: *Agaricus blazei* and *L. edodes* (Carneiro *et al*. 2013). So, the fungi are considered as possible dietary supplements or functional foods against chronic diseases related to oxidative stress. However, when administering them in the diet, it should be kept in mind that during their cooking or processing, their antioxidant activity may be affected (Barros *et al*. 2007). In this work, extracts were heat-treated for 5 min and still maintained antioxidant activity, so it is a good alternative to use as a component of certain enriched foods in which some thermal processes are performed.

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13. IMMUNOMODULATING AND ANTITUMOR PROPERTIES OF *Pleurotus* sp. IN CUBA

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ABSTRACT

In this chapter, we report on the effects of *Pleurotus* (oyster mushroom) extracts (Myc-E and FB-E) and powders (FB-P) on immunodeficient BALB/c mice. The anti-proliferative effect on NB4 human leukemia cells was measured by flow-cytometry. In addition, antioxidant activity was investigated by scavenging of *DPPH* and *ABTS* radicals, reducing power and inhibition of lipid peroxidation. *Pleurotus* mycelial extract (Myc-E) exerted a protective effect in both cyclophosphamide and whole-body irradiated mice in terms of bone marrow cellularity, white blood cell counts and enhancement of the monocyte-macrophage system. Cyclophosphamide treated mice also showed a stimulant effect on cell immune response when administered with fruiting body powder (FB-P). Fruit body-derived extract (FB-E) stimulated immunonutritional recovery of malnourished mice after activation of gut-associated lymphoid tissues. Myc-E reduced viability of NB4 leukemia cells, particularly at a concentration of 200 μg/ml, by arresting cells in the G_2/M phase. At 10 mg/ml, FB-E showed scavenging effects for *DPPH* and *ABTS* radicals (90.4% and 80%, respectively) and inhibited lipid peroxidation (51.2%), whereas at 5 mg/ml manifested a reducing power of 0.438. *Pleurotus* derived-products could be considered good candidates for developing nutraceuticals and innovative myco-therapeutics, as judged by their immunomodulating/antitumor and antioxidant effects.

Keywords: antioxidant, antitumor, immunomodulating, mushrooms, myco-therapeutics, nutraceuticals, *Pleurotus*

INTRODUCTION

Today, the well-being of humankind faces unprecedented challenges involving inadequate regional food supplies, deficiency in new insight into healthy eating, diminishing quality of health, and increasing environmental deterioration (Chang and Wasser 2012). In this context, mushrooms are emerging as a vital component of the human diet and have become attractive as a functional food and as a source of drugs and nutraceuticals (Ferreira *et al*. 2009, Patel *et al*. 2012, Gomes Corrêa *et al*. 2016, Morris *et al*. 2017a).

Fruit bodies as well as mushroom mycelia have a broad range of bioactive properties. Mushrooms are thought to exert approximately 130 pharmacological functions such as antitumor, immunomodulatory, antigenotoxic, antioxidant, anti-inflammatory, hypocholesterolemic, antihypertensive, antiplatelet-aggregating, antihyperglycemic, antimicrobial, and antiviral activities (Lindequist 2013,

Paterson and Lima 2014, Wasser 2014, Prasad *et al.* 2015). These pharmacological effects have been demonstrated for many traditionally used mushrooms, including species from the genera *Ganoderma*, *Lentinula*, *Agaricus*, *Auricularia*, *Flammulina*, *Grifola*, *Hericium*, *Pleurotus*, *Trametes (Coriolus)*, *Schizophyllum*, *Lactarius*, *Phellinus*, *Cordyceps*, *Inonotus*, *Inocybe*, *Tremella*, and *Russula* (Roupas *et al.* 2012, Vikineswary and Chang 2013, Valverde *et al.* 2015).

Several ongoing research projects are directed toward the promotion of mushrooms as a new generation of "biotherapeutics" (Pereira *et al*. 2012, Patel and Goyal 2012). Mycotherapy comprises the use of mushroom-derived extracts and bioactive compounds for their utilization as functional products or drugs with the ability of promoting health. As part of cancer research, mycotherapy is a relatively new and promissory field as a source of agents with immunomodulating and antitumor properties (Popovic *et al.* 2013, Peña-Luna *et al.* 2016). The bioactive molecules comprise high molecular weight compounds, mainly polysaccharides, and low molecular weight secondary metabolites (de Silva *et al*. 2013). Given that only about 10% of mushroom biodiversity has been studied so far, and few of them have been characterized with regard to health benefits, it is likely that new active compounds will be discovered in the future (Hawksworth 2012). Particularly in tropical areas, 22 to 55% (in some cases up to 73%) of mushroom species have not yet been described (Bass and Richards 2011).

Although *Pleurotus* (oyster mushroom) is the second most important mushroom of culinary value (Royse 2014), there has been an upsurge in *Pleurotus* research activities in the last two decades in view of its biopotentialities. This genus includes some of the most popular *Basidiomycetes* edible mushrooms which cultivation has increased greatly throughout the world during the last few decades (Sánchez and Royse 2002, Gomes Corrêa *et al*. 2016). Its popularity has been expanded due to its vigorous growth on a variety of agroforestry substrates and for the production of a high nutritional value-food containing biologically active compounds with therapeutic effects (Carrasco-González *et al.* 2017).

Pleurotus species have been recognized as mushrooms with dual functions to humans both as food and medicine (Khan and Tania 2012, Patel *et al.* 2012). Recent studies on various *Pleurotus* species have shown a number of the pharmacological activities mentioned above (Gregori *et al.* 2007, Deepalakshmi and Mirunalini, 2014, Beltrán *et al.* 2015, Fu *et al.* 2016, Sun *et al*. 2017). In particular, *Pleurotus* spp., are distinguished as important natural resources for immunotherapy, in view of the content of several bioactive compounds able to augment or complement a desired immune response defined as 'host defense potentiators' (HDPs) (El Enshasy *et al.* 2012, Morris *et al.* 2015, Pérez-Martínez *et al.* 2015).

Such bioactive compounds include polysaccharopeptides, polysaccharide-proteins, functional proteins, glucans, proteoglycans and many others. Most of these bioactive compounds follow the immunomodulatory pathway mechanism of mushroom β-glucans by stimulating activities for both innate and adaptive immune systems (El Enshasy *et al.* 2013, Facchini *et al.* 2014). They activate innate immune system components such as natural killer (NK) cells, neutrophils, and macrophages, and stimulate cytokines expression and secretion. These cytokines in turn activate adaptive immunity through the promotion of B cells for antibodies production and stimulation of T-cell differentiation to T helper (Th1 and Th2) cells, which mediate cell and humoral immunities, respectively (Morris *et al*. 2007, Ike *et al*. 2012, Oloke and Adebayo 2015, Tanaka *et al.* 2016).

Both fruit bodies and mycelia of *Pleurotus* spp. have been studied in search of effector molecules (Kyakulaga *et al.* 2013, Morris *et al.* 2012, 2017b). In the opinion of Chang (2001), mycelial products are the "wave of the future" because they ensure standardized quality and year-round production. Thus, submerged liquid fermentation can provide more uniform and reproducible biomass and may afford valuable medicinal products. However, fruit bodies obtained under good manufacturing practice (GMP) can also be used in the formulation of consistent and safe mushroom products such as functional foods, nutraceuticals and biologically active compounds (Morris *et al.* 2014).

Much research work has been reported for various extracts and isolated compounds, particularly polysaccharides, and efforts to find new immunomodulators are ongoing (El Enshasy *et al.* 2013). Therefore, the study of synergy exerted by the vast structural diversity of biomolecules, found in *Pleurotus* crude extracts, powders and other preparations on immune responses, deserves special attention. Better insight into the different roles of multiple active compounds and mechanisms underlying their biological action will accelerate commercial production of pharmaceuticals for therapeutic applications (Figure 1).

In Cuba, implementation of technologies for cultivation of *Pleurotus* spp. on agricultural substrates, in addition to food generation for human consumption, has opened new research activities towards mushroom immunoceuticals. These immunomodulating therapeutic agents can be used in the management of some immunocompromised patients suffering from various diseases, like cancer, HIV/AIDS, liver cirrhosis, acute respiratory failure and recent bone marrow transplants.

The present chapter gives, from an experimental perspective, an updated comprehensive account of some medicinal properties of *Pleurotus* sp. involved in anti-cancer mechanism, such as immunomodulating, anti-proliferative and antioxidant, exerted by extracts and powder preparations obtained from both mycelium and fruit bodies. These results are significant in that they provide a framework for further exploration of the use of *Pleurotus* bioactive preparations for immunotherapy as new life-saving drugs.

MATERIALS AND METHODS

Mushroom material

Pleurotus sp. strain CCEBI-3024 is deposited at the Culture Collection of the Center for Studies on Industrial Biotechnology (CEBI, Cuba). The strain was maintained on slants with solid medium of potato dextrose agar (PDA) incubated at 5 °C.

Preparation of *Pleurotus*-derived products

Pleurotus sp. cultivation was performed by solid-state fermentation of mushroom spawn on pasteurized coffee pulp used as substrate in plastic bags of 2 kg (30-40 cm) (Bermúdez *et al.* 2001). Fruit bodies were harvested, sliced into small pieces and dried at 45 °C for 24 h. Dried material was milled and the resulting powder was preserved away from light and humidity in plastic bags for further use (FB-P). The powder contained 55% (w/w) carbohydrate, 20% (w/w) protein and 4% (w/w) lipids.

Cold water extracts of *Pleurotus* fruit bodies (FB-E) were produced by exhaustively washing the carpophores with distilled water and then slicing them into 1 cm^2 pieces. They were weighed and 5 ml of distilled water was added per g of mushroom tissue. The extraction was made at 20 °C with continuous stirring at 100 rpm for three hours and final extracts were collected by centrifugation and filtration. Extracts were stored at -20 °C and freeze dried. They were mainly composed of 43% carbohydrate and 35% protein.

The preparation of *Pleurotus* mycelium hot-water extract (Myc-E) began with inoculation of mycelium in YPG medium (yeast-peptone-glucose) contained in Erlenmeyer flasks. The flasks were incubated at 27 °C with continuous stirring at 100 rpm for 15 days. After the submerged fermentation was carried out, mycelia were collected by centrifugation at 4,000 rpm and washed twice with distilled water. Isolated mycelia, suspended in 200 g (wet weight)/l of distilled water, were extracted with boiling water for 10 h

and the final extracts were collected by centrifugation and filtration. Extracts were stored at -20 °C and freeze-dried. The major components of Myc-E were carbohydrate (76.8%) and protein (12%).

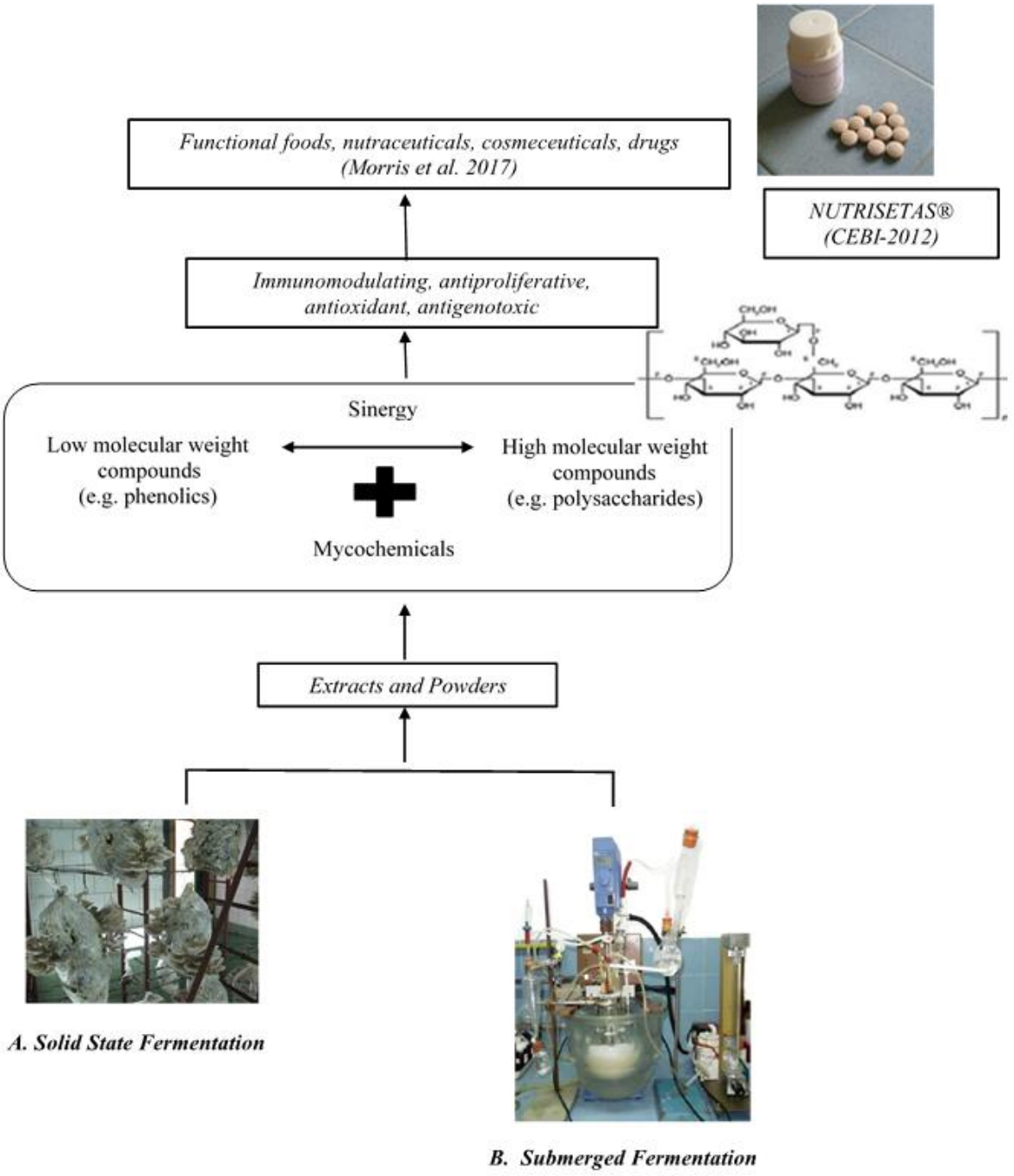


Figure 1. Protocol for developing nutraceuticals and effector molecules with biological activity from *Pleurotus* sp. mushrooms in Cuba.

Laboratory animals

BALB/c mice purchased from the National Center for Production of Laboratory Animals (CENPALAB, Havana) were used. Experiments were carried out under conventional sanitary conditions and animals were maintained at controlled temperature and humidity throughout the investigation ensuring the optimal interval for the specie. The administration of products was made daily in the morning between 9 to 10 am. The research was approved by the institutional Ethical Committee (University of Oriente) and was performed in accordance with Cuban legislation and the National Research Council Guidelines for Care and Use of Laboratory Animals.

Effect of intraperitoneal (i.p.) administration of *Pleurotus* mycelium hot-water extract (Myc-E) to cyclophosphamide-treated or whole-body irradiated mice

Cyclophosphamide (CY)-treated mice.
Fifteen male mice (20-25 g) were divided into two groups. Myc-E was administered intraperitoneally (i.p.) at 100 mg/kg for 7 days to ten Balb/c mice and cyclophosphamide (CY) USP 23 for injection, obtained from JSLYP (China), at 100 mg/kg was given i.p. on the fifth day. The control group, comprised of five mice, was injected i.p. with physiological saline. On the eighth day, blood was collected from the orbital vein and animals were then bled to death.

Whole-body irradiated mice
Male mice were randomly allocated into two groups (n= 10) for eventual whole-body irradiation with a ^{60}Co source Theratron teletherapy unit (Siemens, Erlanger, Germany) in the Oncological Hospital "Conrado Benítez" (Santiago de Cuba, Cuba) at a dose rate of 0.43 Gy/min for 20 min (date of exposure designated as day 0). For the analyses of effects of the mushroom-derived materials, one group of mice was administered with the extract intraperitoneally (i.p.) at a dose of 100 mg/kg in a volume of 0.2 ml on days -10 to -6 and -2 to +1 with respect to the irradiation. Mice in the control group (n=10) were injected with saline solution in place of the extract; non-irradiated mice were used as negative controls. All mice were euthanized 24 h after the final administration of extract or saline, and tissues/blood were isolated for analyses.

In both experiments, blood specimens were analyzed for white blood cell count. Femoral bone marrow cells were withdrawn with Hanks' solution and counted with a Neubauer chamber (Germany). Spleen cell suspensions were prepared by gently teasing the tissue with ice-cold Hanks' solution and passing it through antiseptic gauze (Johnson & Johnson Medical, TX, USA) and counted with a Neubauer chamber. Peritoneal exudate cells were collected from the peritoneal cavity of mice by washing with Hanks' solution and also counted.

Functional activity of the monocyte-macrophage system of each host was evaluated using a carbon clearance test. The clearance rate of carbon was expressed as the ratio of absorbance for samples from Myc-E treated (or saline-control) mice with respect to values from immunocompetent mice injected with carbon particles (i.e., received no test substances) (see Morris *et al*. 2008 for details).

Effect of oral administration of *Pleurotus* sp. fruit bodies powder (FB-P) on cell immune response of cyclophosphamide treated mice

The 20 to 25 g male BALB/c mice were fed a standard diet and acidified water *ad libitum*. Fifteen mice were divided into three groups (n= 5). The two experimental groups were treated intraperitoneally (i.p) with cyclophosphamide (CY) USP 23 for injection obtained from JSLYP (China) at 100 mg/kg on day 0. FB-P was administered by oral route (1,000 mg/kg) for 7 days to the 'CY/ FB-P¨ group, whereas

physiological saline solution was administered to the 'CY/ Saline' group in a similar schedule. Non-treated mice were used as controls in the experiment.

The effect of FB-P on cell-mediated immunity was determined by the delayed-type hypersensitivity (DTH) reaction (Kim *et al.* 1998). Mice were immunized by an intradermal (i.d.) injection of 50 µl of 5 mg/ml BSA emulsified in Complete Freund Adjuvant (CFA; Sigma, St. Louis, MO, USA) at two sites on the abdomen. Eight days after immunization, mice were re-challenged by injection of 20 µl of 5 mg/ml BSA into one rear foot pad, while the other received a comparable volume of phosphate buffered saline (PBS). Measurements of foot pad swelling were taken at 24, 48 and 72 h after challenge by use of a micrometer (Mitutoyo, Tokyo, Japan). The magnitude of the DTH response was determined as differences in foot pad thickness between the antigen and PBS-injected foot pads. A similar immunization protocol was applied to control animals. Histological studies were made with samples taken from the antigen-injection sites with the hematoxylin-eosin staining and observations performed under an optical microscope (100x).

Popliteal lymph nodes (right and left) of antigen sensitized and re-challenged animals were removed and washed with PBS pH 7.4. Excess moisture was discarded with a filter paper and the lymph nodes were immediately weighed separately with an electronic analytical balance (Sartorius). The mass index was expressed as the relation between weights of the popliteal node belonging to BSA-injected foot pad with respect to that of PBS-injected pad (Descotes 2006).

Effect of oral administration of *Pleurotus* sp. fruit bodies cold water-extract (FB-E) to malnourished BALB/c mice

Female BALB/c mice, weighing 20 g, were housed individually at 23 °C with a 12-hour/12-hour light/dark cycle. Thirty mice that were starved for 3 days and had free access to salted water were studied. After this time, blood was collected from the orbital vein of 10 mice and the animals were killed (M group). The others were re-fed *ad libitum* for 8 days with commercial pelleted diet (M-CD group) or with the commercial diet and the *Pleurotus* fruiting bodies cold water-extract (FB-E) administered orally at a dose of 100 mg/kg of body weight per day (M/FB-E group). A control group of 10 mice was fed with commercial diet throughout the study.

After the small intestine was collected, the segment correspondent to jejunum was rinsed thoroughly with ice-cold saline solution, opened, and blotted dry. Mucosa was scraped with a glass slide and weighed separately. Jejunal mucosa was homogenized with ice-cold phosphate-buffered saline with a pH of 6.0 (1:3 w/v). Total protein and DNA were estimated by the methods of Lowry *et al.* (1951) and Burton (1956), respectively.

Functional activity of the monocyte-macrophage system was evaluated as described previously for cyclophosphamide-treated or whole-body irradiated treated with Myc-E.

Humoral immune response was evaluated through an immunization protocol with sheep red blood cells (SRBC) as antigen. Three groups, comprised of five mice, were designed: M-DC, M/FB-E and control as described above. After the starvation (day 0) mice were injected intraperitoneally (i.p.) with 0.2 ml of a 25% SRBC saline solution. After 7 days from the first injection, blood samples of 50 µl were drawn from the orbital plexus to measure antibody titres by a haemagglutination (HA) reaction. Reciprocal serum dilution, which just gave agglutination, was considered the titre. At this time, mice received the second immunization and on day 14, antibody titres were determined.

Cytometric analysis of cell viability of human acute promyelocytic leukemia (NB4) cells

Cells were maintained in RPMI medium (Gibco-Life Technologies) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.02 mg/ml gentamycin. Cells were cultured at 37°C in a humidified air atmosphere with 5% CO_2. Cell viability of NB4 cells was determined by flow cytometry by measuring the level of impermeability to propidium iodide (PI). Briefly, 5×10^5 cells were seeded in a 12-well culture plate (Falcon, Becton Dickinson, NJ, USA) 24 h prior to treatments. Then, NB4 cells were incubated for 24 h with Myc-E extract at doses of 100 and 200 μg/ml; cells incubated with culture medium alone served as controls. After incubation, cells were collected and washed in PBS and centrifuged at 1200 rpm for 5 min. The cells were resuspended in 500 μl of PBS and stained with PI to a final concentration of 50 μg/ml and analyzed in a FACS caliber flow cytometer (Becton Dickinson, San José, CA, USA). Data analysis was performed using WinMDI 2.8 software (Trotter 2011).

For analysis of cell-cycle distribution of NB4 cells, 5×10^6 treated cells were centrifuged at 1500 rpm for 3 min. Then, they were resuspended in 500 μl PBS containing 0.1% Nonidet P-40 and 0.5 mg/ml DNAse-free RNAse A. Cell suspensions were incubated at room temperature for 30 min in order to extract low molecular weight DNA from cell nuclei. Remnant DNA in cells was stained with 50 μg/ml PI and immediately measured in the cytometer. Cell-cycle progress was expressed as the percentage of cells in G_0/G_1, S, and $G_{2/}M$ phases. Histograms of untreated cells were used to define positions of different phases of the cell cycle.

Assays for *in vitro* antioxidant activity with FB-E extract

DPPH assay

Radical scavenging ability (RSA) of mushroom extract against 1,1-diphenyl-2-picryl-hydrazyl (DPPH, Sigma-Aldrich) was measured according to Cheung *et al.* (2003) using spectrophotometry. FB-E (1 ml) at 10 mg/ml was mixed with 0.5 ml of 0.1 mM DPPH ethanolic solution. Then, the mixture was shaken vigorously and incubated at 25°C for 1 h in the dark. The absorbance of the sample was measured at 520 nm (VIS-723G spectrophotometer, Beijing Rayleigh Analytical Instrument Corporation) and the scavenging ability against DPPH radicals was calculated as a percentage of DPPH discoloration using the equation: % RSA = $[(A_{DPPH} - A_S)/A_{DPPH}]$ x 100, where A_S is the absorbance in the solution when the sample extract has been added, and A_{DPPH} is the absorbance of DPPH solution. L-ascorbic acid was used as a standard.

ABTS assay

Scavenging effect on 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich) radicals was measured according to Choi *et al.* (2006). ABTS radicals were generated by mixing 7 mM of the ABTS stock solution and 2.45 mM of potassium persulfate (Sigma-Aldrich) in distilled water, and storing this mixture overnight at room temperature in the dark. The mixture (10 ml) was diluted with 840 ml of distilled water. *Pleurotus* extract (50 μl) at 10 mg/ml was added to 3 ml of ABTS solution and after 90 min, the absorbance was measured at 414 nm (VIS-723G spectrophotometer, Beijing Rayleigh Analytical Instrument Corporation). The scavenging ability against ABTS radicals was calculated using the equation: % RSA = $[(A_{ABTS} - A_S)/A_{ABTS}]$ x 100, where A_S is the absorbance in the solution when the sample extract has been added, and A_{ABTS} is the absorbance of ABTS solution. L-ascorbic acid was used as a standard.

Reducing power

The reducing power was determined according to the method of Lee *et al.* (2007). Mushroom extract FB-E (2.5 ml) at 5 mg/ml was mixed with 2.5 ml of 10 g/l potassium ferricyanide (Sigma-Aldrich) and the mixture was incubated at 50°C for 20 min. Then 2.5 ml of 100 g/l trichloroacetic acid (Merck) was added,

and the mixture was centrifuged at 2 000 g for 10 min. A sample of the supernatant (5 ml) was mixed with 5 ml of distilled water and 1 ml of 1 g/l $FeCl_3$ (Merck), and the absorbance was measured at 700 nm (VIS-723G spectrophotometer, Beijing Rayleigh Analytical Instrument Corporation). Butylated hydroxyltoluene (BHT) was used as a standard.

Determination of inhibitory activity on lipid peroxidation.
A reaction mixture containing 8 ml of a suspension of 20% sheep erythrocytes, 7.52 ml of physiological saline, 80 µl of 0.5 mol/l $FeCl_3$, 0.4 ml of ascorbic acid (0.5 mM) and 1 ml of FB-E (10 mg/ml) was incubated at 37 ^oC in a water bath for 120 min. The lipid peroxide formed was estimated by measuring thiobarbituric acid reacting substances (TBARS) with some modifications (Okhawa *et al.* 1979). For this, 2 ml of the incubation mixture was treated with 1 ml of trichloroacetic acid at 10%, the samples were shaken in a vortex for 1 min and centrifuged at 6 000 g for 15 min. Then, 2 ml of the supernatant was transferred to test tubes with 2.5 ml of 0.335% thiobarbituric acid (TBA) and the reaction tube was kept in a water bath at 100^oC for 1 h. After cooling, the absorbance was measured at 532 nm. The percentage of inhibition of lipid peroxidation was determined by comparing the results of the test compounds (treated with mushroom extract) with those of control (not treated with the mushroom extract). The percentage of lipid peroxide-scavenging ability of the extract was calculated by the formula described in DPPH and ABTS radicals scavenging effect.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). One-way analysis of variance and *post hoc* Tukey's tests or Kruskal-Wallis rank test followed by the Student-Newman-Keuls test was applied to determine the significance of differences between treatments. The Student's *t*-test or Mann-Whitney´s *U*-test was used to compare the two means in the experiments related to the effects of extracts in cyclophosphamide-treated or whole-body irradiated mice. Differences at $p< 0.05$ were accepted as significant. The software Statgraphics Plus v. 5.1 (Statistical Graphics Corporation, 1994-2001) was used in the analysis.

RESULTS AND DISCUSSION

Cancer is a worldwide disease that is causing serious damage to human health. How to conquer cancer is one of the most important research topics in medicine. The immune system is the human's ultimate defense against infectious diseases, tumors, and cancer growth (El Enshasy and Hatti-Kaul 2013). Minimal toxicity and potent biopharmacological activities of mushroom metabolites has received increasing attention in cancer therapy. Recently, numerous reports have been published on preclinical studies and clinical trials related to the functionality and bioactive properties of *Pleurotus* mushrooms and their nutraceutical derivatives, including immune modulatory and antitumor effects (Pérez-Martínez *et al.* 2015, Xu *et al.* 2016, Carrasco-González *et al.* 2017).

Although in our study polysaccharides appear to be the most important bioactive component in *Pleurotus*-derived preparations with respect to immunomodulation, the presence in varying amounts of different secondary metabolites could lead to a synergy for immune enhancing activity. Results of mycochemical tests showed that both *Pleurotus* fruit bodies and mycelial extracts contain alkaloids, phenolic compounds like flavonoids and tannins, reducing sugars and amino acids (Morris *et al.* 2014). Moreover, fungal immunomodulatory proteins purified from medicinal mushrooms, comprise a group of novel proteins that possess immunomodulatory properties and have a strong potential of being applied to food or pharmaceutical products for commercial development (Ou *et al.* 2009). Differences in biosynthesis patterns of cell molecular components in distinct stages of the vital cycle would explain the dissimilarities in biochemical composition of fruit bodies and mycelial extracts.

Immunomodulating effects of Myc-E in cyclophosphamide-treated or whole-body irradiated mice

Chemotherapy and radiotherapy in cancer treatment contribute to further depression of the immune system. Use of immunomodulating therapeutic agents can help to minimize these problems and efforts to find new immunomodulators are on-going (Zhuang 2009). For that reason, we studied the effects of intraperitoneal administration of *Pleurotus* mycelium hot-water extract (Myc-E) to cyclophosphamide-treated or whole-body irradiated mice.

Cyclophosphamide (CY) is currently one of the most widely prescribed alkylating agents in cancer chemotherapy; however, CY treatment often results in potent immunosuppressive and cytotoxic effects (Morris *et al.* 2003). Immunosuppression caused by CY and other anticancer drugs significantly complicates the course of cancer chemotherapy and worsens the condition of patients (Hou *et al.* 2007). As expected, cyclophosphamide severely impaired mice hematopoietic tissue, but Myc-E was found to have an active protective effect. Myc-E increased bone marrow cellularity and white blood cell counts compared to the CY-saline control group ($p< 0.05$) (Figure 2) and no significant increase was observed in spleen cellularity. On the other hand, the radioprotective effect exerted by mycelium Myc-E was evident by increases in bone marrow cellularity, leukocyte counts and in spleen cellularity ($p< 0.05$), compared with the irradiated control group (Figure 2). Stimulation of production of white blood cells by bone marrow in an immunosuppressed animal model has been classified as an immunomodulatory effect (Gupta *et al.* 2010).

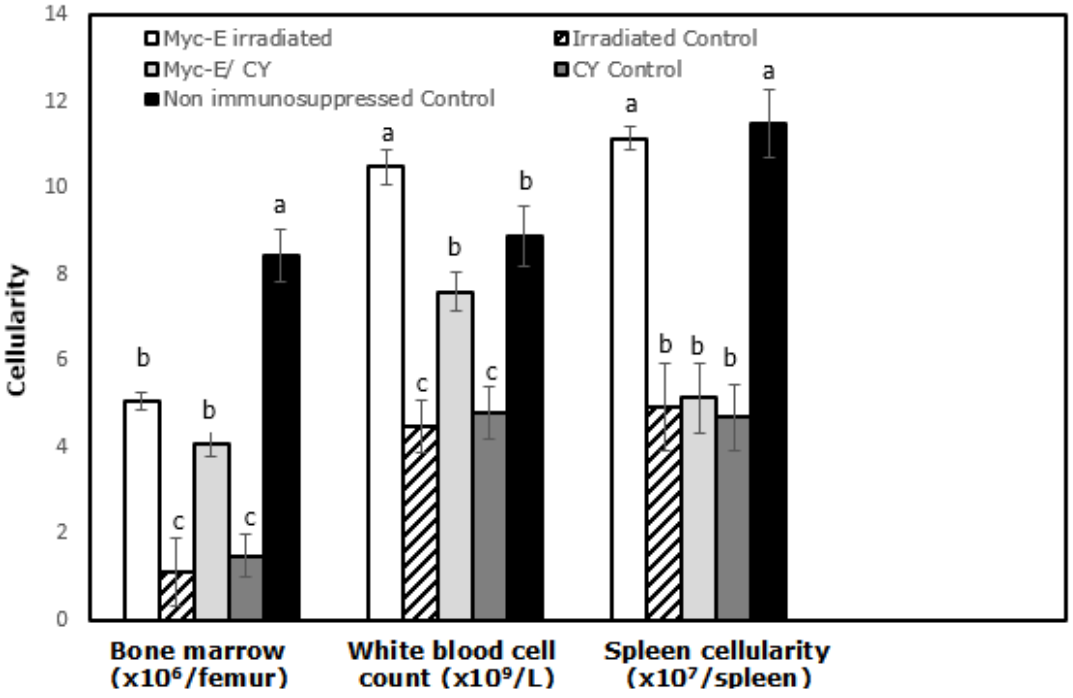


Figure 2. Effects of *Pleurotus* sp. mycelial extract (Myc-E) on haemopoiesis of cyclophosphamide-treated or irradiated BALB/c mice. Values shown are means (±SE) of each group (n= 10). Different letters indicate significant differences among groups (Kruskal–Wallis, Student–Newman–Keuls, $p< 0.05$).

Since macrophages have been suggested to play important roles in immunological surveillance, we studied the influence of administration of Myc-E on the number of peritoneal exudate cells and phagocytic activity of macrophages (Table 1). In the CY-treated or irradiated mice, immunosuppression manifests as a markedly decrease in the numbers and phagocytic activities of macrophages. Myc-E at 100 mg/kg remarkably increased the number of peritoneal exudate cells compared with saline control groups in both immunosuppression models ($p< 0.05$). In the study to evaluate effects of the extract on *in vivo* phagocytic activity by measuring carbon clearance in peripheral blood (as an index of the phagocytic activity of liver and spleen), a low ratio was deemed to correspond to a high clearance of carbon from the blood. Data show that treatment with Myc-E extract potentiated activity of the host monocyte-macrophage system (relative to that in the CY or irradiated saline treated mice) (Table 1).

Table 1. Effects of *Pleurotus* sp. mycelial extract (Myc-E) on the number of peritoneal exudate cells and macrophage phagocytic activity of cyclophosphamide-treated or irradiated BALB/c mice.

Experimental groups	Number of peritoneal exudate cells (x 10^6/mouse)	Macrophage phagocytic activity (Absorbance ratio at 5 min)
Myc-E/ irradiated mice	4.61 ± 1.43 a	1.62 ± 0.12 b
Irradiated-saline control	1.82 ± 0.65 b	2.01 ± 0.31 a
Myc-E/ CY treated mice	4.9 ± 1.4 a	1.67 ± 0.11 b
CY-saline control	2.9 ± 0.1 b	1.98 ± 0.02 a
Non-immunosuppressed control	3.41 ± 0.57 a	-

Values are means ± SE, n= 10. Different letters indicate significant differences, Student's *t*-test, $p< 0.05$)
For number of peritoneal exudate cells, comparisons were made with respect to non-immunosuppressed mice and for phagocytic activity between both irradiated or CY-treated animals.
(-) The value was used in estimation of absorbance ratios.

These results were in keeping with the finding of another study wherein water-soluble fractions *of P. ostreatus* mycelium exerted modulating effects on macrophage activation *in vitro*, as reflected in enhanced glucose consumption and acid phosphatase activity by treated cells (Morris *et al.* 2007). Immunomodulatory effects of Myc-E in *in vitro* systems have been investigated (murine macrophages RAW 264.7). The extract can induce functional activation of macrophages by inducing nitric oxide (NO) release and increasing mRNA expression of inducible nitric oxide synthase (iNOS) (Llauradó *et al.* 2016). These results confirm the vital role of Myc-E in triggering immune responses.

Noted increases in macrophage activation might be related to binding of one or more extract components to receptors found on macrophage surface such as glucan receptors (e.g. dectin-1). Polysaccharides appear to be the most important component with respect to antitumor effect and on average, 1.5% of Myc-E dried mass consists of β-1,3-1,6-glucans (Morris *et al.* 2014).

Pillai and Uma Devi (2013) reported an increase in the survival index (66%) and an improvement in hepatic function and haematological parameters in bone marrow of irradiated mice (6 and 8 Gy) treated with *Ganoderma* preparations. These findings agree with those of a clinical trial wherein patients with

different types of cancer (hepatic, lung, gastric, colorectal and nasopharyngeal) who were undergoing chemotherapy or radiotherapy received a nutritional supplement containing polysaccharides extracted from six different mushrooms (Novaes and Fortes 2005).

Reversing the function of immune suppressed cells may improve efficacy of cancer therapy (Cui *et al.* 2015). Hence, Myc-E may be a candidate therapeutic agent with chemo- and radioprotective activity for hematopoiesis damage, particularly to cells involved in immune function. Although other studies described the radioprotector effect on the immune system of mushroom products (Guggenheim *et al.* 2014), application of Myc-E (from *Pleurotus* mycelium) in immune suppression research appears to be new as reflected in the literature. These data could be complemented by further experiments, possibly investigating also cytotoxicity exerted by the extract on tumor cell lines, as shown later in this chapter.

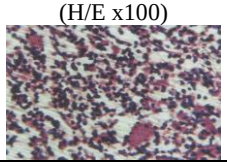
Immunoenhancing activity of FB-P on cyclophosphamide treated mice

In vivo efficacy of *Pleurotus* preparations in immunological effector cells that have a key function against tumor growth under immunosuppression, is poorly understood. Most of the *in vivo* results come from studies with a polysaccharide injection, rather than oral administration (Wang *et al*. 2014). Polysaccharides enriched-extracts that elicit effects *in vitro*, or by injection, may be ineffective or may exhibit different effects when taken orally (Boh *et al.* 2007). Evidence of the effectiveness of oral mushroom preparations against immune challenges must be ultimately demonstrated in animals. Moreover, well-characterized formulations must be developed based on a complete understanding of the immunomodulatory effects and specific applications of oral myco-products. Thus, dried and powdered *Pleurotus* mushroom (FB-P) could become an attractive alternative for the development of functional foods and nutraceuticals preparations.

In the present section, activation of FB-P on cell immune response was evaluated *in vivo* on cyclophosphamide treated mice. Results indicated that none of the FB-P-orally treated mice died and their body weights showed no significant change during the course of the experiment ($p< 0.05$, data not shown). Mice supplemented with *Pleurotus* powder (FB-P) showed a higher delayed-type hypersensitivity (DTH) response, as judged by the increase of foot pad swelling compared to the saline control group, particularly at 48 and 72 h after antigen re-challenge ($p< 0,05$; Table 2). DTH response mounted at these times by the CY-FB-P group was similar to that of control mice. Reconstitution of DTH response reflected induction of $CD4^+$ Th1 cells and the activation of macrophages by cytokines: tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) (Kim *et al.* 1998, Murphy and Weaver 2016). Mononuclear cells were shown infiltrating antigen-injection sites in CY/FB-P treated mice (Figure inserted in Table 2). In addition, DTH reconstitution was associated with the increase observed in the mass index of popliteal lymph nodes of FB-P supplemented animals ($p< 0.05$; Table 2). In sum, results evidenced that FB-P reversed CY-induced damage on adaptive cell immunity and promoted proliferation of T cells and macrophages.

It has been demonstrated that a polysaccharide of *P. citrinopileatus* (PCPS) has the capacity to activate human dendritic cells (DCs) via multiple pathways, leading to secretion of pro-inflammatory cytokines TNF, IL-1β, IL-6 and IL-12, as well as anti-inflammatory cytokine IL-10 (Minato *et al.* 2016). As is known, activated macrophages, NK cells, cytotoxic T cells and their secretory products, such as tumor necrosis factor, reactive nitrogen and oxygen intermediates and interleukins are involved in immunomodulatory responses (Wang *et al.* 2015).

Table 2. Effect of oral administration of FB-P on cell immune response (DTH response) of cyclophosphamide treated BALB/c mice.

Experimental groups	Foot pad thickness (mm)			Mass index of popliteal lymph nodes	CY/ FB-P 48 h (H/E x100)
	24 h	48 h	72 h		
Control	0.48 ± 0.07^{a}	0.46 ± 0.04^{a}	0.38 ± 0.07^{a}	-	
CY/ FB-P	0.14 ± 0.05^{b}	0.43 ± 0.02^{a}	0.29 ± 0.05^{a}	1.87 ± 0.27*	
CY/ Saline	0.11 ± 0.06^{b}	0.39 ± 0.01^{b}	0.12 ± 0.04^{b}	1.34 ± 0.15	

All values are expressed as the arithmetic mean ± S.D. of five mice. Different letters indicate significant differences among groups (Kruskal-Wallis, Student-Newman-Keuls, $p < 0.05$). The (*) reflects significant differences between the two groups in the Mann-Whitney test ($p < 0.05$).
Mononuclear cells infiltrating antigen-injection sites in CY/FB-P are shown in the inserted figure (hematoxylin-eosin staining, 100x).

Oral administration of *P. nebrodensis* polysaccharide (PN-S) to CY-immunosuppressed mice increased thymic and splenic indices and promoted proliferation of T lymphocyte, B lymphocyte and macrophages. PN-S also enhanced activity of natural killer cells and increased immunoglobulin M (IgM) and immunoglobulin G (IgG) levels in serum. PN-S also increased levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ) and nitric oxide (NO) in splenocytes. These results suggest that PN-S treatment enhances immune function of immunosuppressed mice. This study may provide a basis for application of this fungus in adjacent immunopotentiating therapy against cancer and in treatment of chemotherapy- induced immunosuppression (Cui *et al.* 2015).

Mariga *et al.* (2014) reported that *P. eryngii* fruit bodies powder is an active antitumor agent with immunomodulatory activity, where, it targets lysosomes of cancerous cells concomitantly stimulating macrophage-mediated immune response. Based on current data, we demonstrated that *Pleurotus* sp. mushrooms might be of potential benefit in anticancer-drug induced immunosuppression. Our findings suggest that oral administration of *Pleurotus* sp. powder would stimulate the immune system after their absorption in the gastrointestinal tract and the activation of gut-associated lymphoid tissues, thus integrating different elements of immune function (see results on the animal model of malnutrition).

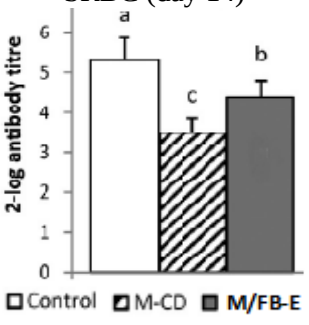
Immunonutritional restoration effects of FB-E on malnourished BALB/c mice

In protein-energy malnutrition (PEM), there are reductions in nutrient absorption and depression of the immune system in gut-associated lymphoid tissues (Schaible and Kaufmann 2007). Functionality and integrity of the small intestine also deteriorated. Administration of FB-E to malnourished mice during the refeeding period provided beneficial effects and improved functional alterations in the intestinal tract ($p < 0.05$) (Table 3). The increase of mucosal weight in association with high values of protein and DNA content suggests a higher rate of protein biosynthesis and could be related to the recovery of gastrointestinal tract function. Some components present in *Pleurotus* mushrooms could re-establish intestinal architecture after oral consumption.

In general, enteral nutrition is considered the first method of feeding in critical patients (Botrán and López-Herce 2011) and mushroom substances might be potential candidates for use in immunonutritional diets. Some studies with mushrooms reported the biological potential of polysaccharides at the intestinal level by means of stimulation of gut-associated lymphoid tissues and intestinal macrophages (Bouike *et al.* 2011).

Table 3. Effect of oral administration of FB-E on immunonutritional restoration of malnourished BALB/c mice with respect to gut mucosa and humoral immune response.

Experimental groups	Gut mucosa parameters		Antibodies titres against SRBC (day 14)
	Protein (μg/ 10 cm)	DNA (μg/ 10 cm)	
Control	352 ± 78^{b}	59 ± 16^{b}	
Malnourished mice (M)	108 ± 38^{c}	45 ± 20^{b}	
M/ CD	277 ± 76^{b}	68 ± 6^{b}	
M/ FB-E	576 ± 87^{a}	98 ± 21^{a}	



Results are expressed per ten centimeters of intestine. All values are the arithmetic mean ± SD of 10 mice. Different letters indicate significant differences among groups (Kruskal-Wallis, Student- Neuman-Keuls, $p< 0.05$). Subscript legend: (a) ≠(b) ≠ (c).

Oral administration of FB-E at a dose of 100 mg/kg stimulated phagocytic activity in comparison with the standard diet group. The ratio of carbon clearance at 5 min was lower in FB-E group than in the M-CD group (1.4 ± 0.1 *vs*. 1.9 ± 0.1) ($p< 0.05$); a low ratio was deemed to correspond to a high clearance of carbon from the blood. Nevertheless, FB-E does not increase spleen weight and splenic cell counts ($p< 0.05$) (data not shown). Augmentation of phagocytic activity may be owing to activation of phagocytes and not by an increase in the number of total phagocytes. Effects on the intestinal tract linked to macrophage activation might be influenced by a partial absorption of bio-compounds from *Pleurotus* sp., or by stimulation of gut-associated lymphoid tissues. Nevertheless, the mechanism(s) of action of several orally administered bio-substances from mushrooms is still unclear. Among various myco-chemicals, it has been suggested that only fragments of polysaccharides partially hydrolyzed or degraded after ingestion might bind to gut epithelia and exert localized and/or systemic effects on the immune system or the mechanisms could be mediated via a non-specific intestinal absorption (Wasser *et al.* 2014). Although most of the bio-components in FB-E could be implicated as immunomodulatory agents, more evidence is required to link observed actions to any of the identified bio-components.

Although cell-mediated immunity is severely affected in PEM, atrophy of lymphoid tissues leads to a decrease of circulating and splenic B cell numbers (Lykke *et al.* 2013). However, the role of humoral immune response in malnourished mice is not well documented. Antibody production by B cells after 14 days of immunization with sheep red blood cells (SRBC) was significantly higher compared with mice refed with commercial diet ($p< 0.05$) (Figure inserted in Table 3). Anti-SRBC antibodies (directed against a T-dependent antigen) titres might also suggest stimulation of cellular immunity. Other studies indicated that the humoral response might respond to malnutrition, depending on malnutrition type (e.g. acute *vs.* chronic, protein malnutrition *vs.* energy restriction) (Neyestani *et al.* 2004).

The term *immunonutrition* was introduced as emergent subject in the last few years (Zapatera *et al.* 2015). In this context, FB-E could be used to develop specific enteral formulations with potential applications in immunotherapy and as immunonutritional support during recovery of metabolic and immunological disorders associated with malnutrition. This study is a contribution to the knowledge of the immunonutritional properties of *Pleurotus* mushrooms and suggests its prospective use in immunocompromised people with special nutritional requirements.

Antiproliferative effects of Myc-E on human acute promyelocytic leukemia (NB4) cells

Since research has tended to focus on the dietary value of species of the genus *Pleurotus*, there is relatively little information pertaining to their anticancer mechanisms, particularly in mycelia-derived products. Treatment with *Pleurotus* mycelial extract (Myc-E) reduced viability of NB4 human leukemia cells, particularly at a concentration of 200 μg/ml, to 82% relative to control nontreated cells ($p< 0.05$) (data not shown).

Our results presented in Figure 3 indicated that the cytotoxic effect of a hot water extract from *Pleurotus* sp. mycelia on NB4 cells is related to its ability to arrest the cell cycle. Independent of concentration, the extract lowered leukemic cells in the G_0/G_1 phase compared to untreated cells ($p< 0.05$), but had no significant effect on the S population. Overall, *Pleurotus* sp. mycelial extract significantly increased the number of NB4 cells in G_2/M phase (15.82% and 18.35% for cells treated, respectively, with 100 and 200 μg/ml, *vs* 8.78% in control cells, $p< 0.05$). Thus, *Pleurotus* sp. extract arrested NB4 cells in the G_2/M phase supporting a cell-cycle dependent anticancer mechanism.

While a water-soluble non-starch polysaccharide extracted from mycelium (EDP) of *P. tuber-regium* caused G_2/M arrest in HL-60 cells by lowering Cdk1 expression, its fruiting-body analog (HWE) caused S arrest in HL-60 cells by a depletion of Cdk2 and an increase in cyclin E expression (Wong *et al.* 2007). This was in contrast to a previous study of a β-glucan obtained from *Poria cocos* mycelium that was found to inhibit proliferation of MCF-7 cancer cells by G_1 arrest and apoptotic induction via down regulating anti-apoptotic protein Bcl-2 (Zhang *et al.* 2006).

On the other hand, the effect of several extracts of various polarities obtained from fruiting bodies of *Pleurotus* sp. was tested on apparent growth of different cell lines (U937, N2A and Caco2 tumoral cells compared to Vero cells). *In vitro* growth activity of cells with aqueous (CW-P at 4°C and HW-P at 100°C), methanol (MetOH-P) or dichloromethane (DM-E) extracts were estimated through mitochondrial activity using an MTT (a Tetrazolium salt) Test and by Neutral Red Uptake assays. Inhibition of cell respiration and cell uptake was observed with CW-P extracts while HW-P and MetOH-P extracts were less efficient. Compared to HW-P, substance(s) responsible for this effect in CW-P appeared thermo-labile. However, cell proliferation was shown in U937 with intermediate dilutions of HW-P. N2A was specifically sensitive to inhibition by MetOH-P extract (Llauradó *et al.* 2014). The mechanisms by which they drive benefits remain obscure, while the effective doses and their safety need to be evaluated.

Dietary supplements of edible *Pleurotus* spp. rich in fungal polysaccharides is associated with anticancer health benefits. Tong *et al.* (2009) isolated a novel water-soluble polysaccharide (POPS-1) from fruit bodies of *P. ostreatus* by hot water extraction. Cytotoxicity assay showed that POPS-1 presented significantly higher antitumor activity against Hela tumor cells *in vitro*, in a dose-dependent manner, and exhibited significantly lower cytotoxicity to human embryo kidney 293T cells than Hela tumor cells. These results suggest that *P. ostreatus* water-soluble preparations may be considered as potential candidates for developing new low toxicity antitumor agents.

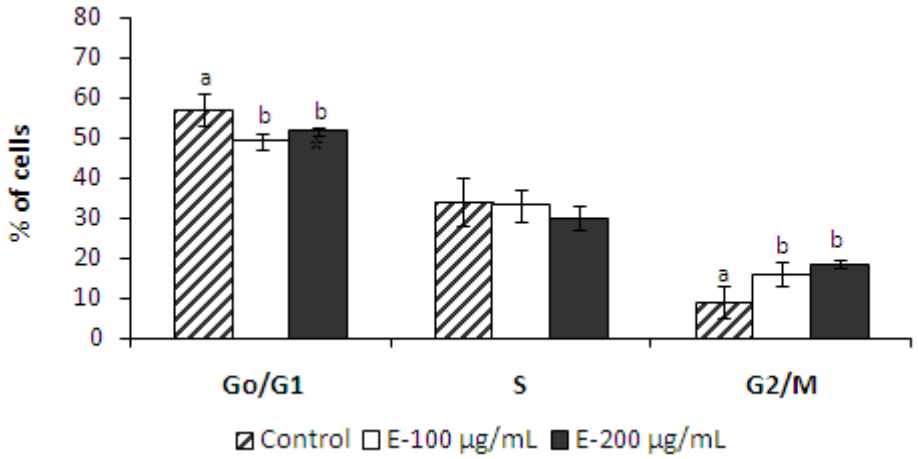


Figure 3. Cell-cycle analysis of human leukemia NB4 cells treated with *Pleurotus* sp. mycelial extract Myc-E for 24 h at concentrations of 100 and 200 µg/ml. Values represent the mean ± SE of three independent experiments. Significantly differences in Tukey's test ($p< 0.05$) were shown with different letters compared to control cells. Non-significant differences were found in the S-phase of cell-cycle.

In vitro antioxidant activities

Free radicals are known to be a major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer. Antioxidant compounds play an important role in preventing and curing chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Sun *et al.* 2017). The antioxidant potential of a compound could be attributed to its various characteristics; the most important of these is the ability to scavenge and reduce free radicals, to chelate transition metal ions and to inhibit lipid peroxidation, among others (Rajeshwar *et al.* 2005).

The antioxidant effects displayed by edible mushrooms, in addition to their immunomodulating properties represent an important contribution to their antitumor activities (Patel and Goyal 2012). Antioxidant properties of *Pleurotus* spp. were of both enzymatical and non-enzymatical nature (Khatun *et al.* 2015). Until now, research has tended to focus on the dietary significance of edible mushrooms; however, there is relatively little information relating to antioxidant activity and possible use of such mushrooms to neutralize oxidative stress (Jayakumar *et al.* 2011).

Antioxidant activities of FB-E determined using four *in vitro* assays are presented in Table 4. DPPH is a stable free radical that shows maximum absorbance at 517 nm in methanol; when DPPH encounters a proton-donating substance such as an antioxidant, the radical is scavenged and absorbance is reduced (Kohen and Nyska 2002). As shown in Table 4, the DPPH radical scavenging activity of the aqueous extract obtained from *Pleurotus* fruit bodies was 90.4%, lower than values achieved with ascorbic acid used as control. In previous studies, a DPPH radical scavenging ability of 96% was reported with a mycelial extract (Morris *et al.* 2017b). Scavenging effects of extracts from several specialty and commercial mushrooms on DPPH radicals augmented with increased extract concentrations. Thus, DPPH radical scavenging activity varied from 9% (*P. nebrodensis*) to 57% (*P. cystidiosus*). Moreover, DPPH scavenging action for *P. citrinopileatus* and some other fungi significantly improved with gradual elevation of sample concentration from 0.5 to 9.0 mg/ml (Asatiani *et al.* 2010).

Table 4. *In vitro* antioxidant activity of a water extract of fruit bodies of *Pleurotus* sp (FB-E).

Sample	Scavenging of DPPH radicals (%)	Scavenging of ABTS radicals (%)	Reducing power ($A_{700\ nm}$)	Inhibition of lipid peroxidation (%)
Pleurotus extract (FB-E)	90.4 ± 0.8 (10 mg/ml)	80 ± 0.9 (10 mg/ml)	0.438 ± 0.034 (5 mg/ml)	51.2 ± 4.8 (10 mg/ml)
Control	96.3 ± 0.6* (ascorbic acid)	98 ± 0.2* (ascorbic acid)	0.700 ± 0.018* (BHT)	92 ± 1.4* (ascorbic acid)

Results are showed as means ± standard deviation of three replicates; means with an (*) differ when compared with the *Mann–Whitney* test ($p< 0.05$).

With the ABTS radical scavenging test, we can measure activity of both hydrophilic and lipophilic compounds; therefore, it is useful in the simultaneous study of several natural ingredients (Kuskoski *et al.* 2005). In this study, ABTS radical scavenging activity of FB-E mycelium at 10 mg/ml was of 80%, higher than a mycelial extract with a value of 55% (Morris *et al.* 2017b). On the other hand, hydro-alcoholic extracts of *Grifola gargal* showed an ABTS radical scavenging ability of 90.9-93.3 ascorbic acid equivalents (mg of ascorbic acid per l of sample) (De Bruijn *et al*. 2009). This method also has been used in evaluation of antioxidant activity of neutral polysaccharides from *Auricularia auricula* and their homologues sulfated in concentrations of 0.2-10 mg/ml, without significant differences (Zhang *et al.* 2011).

Moreover, it has been discussed that the diphenylpropane structure of flavonoids and the aromatic ring structure of phenolics, such as aromatic oxy phenol acids, might contribute to free radical scavenging ability of these compounds (De Bruijn *et al*. 2008).

Reducing capability of a compound may serve as a significant indicator of its potential antioxidant activity and the efficacy of certain antioxidants is known to be associated with their reducing power (Lü *et al.* 2010). In the present study, reducing power of a 5 mg/mL concentration of mushroom extract was 0.438, which was relatively lower than that of BHT ($p< 0.05$) (Table 4). The reducing power of medicinal mushrooms might be due to their hydrogen-donating ability (Jayakumar *et al.* 2011). Possibly, medicinal mushrooms contain high amounts of reductones that could react with radicals to stabilize and terminate radical chain reactions. Reducing power of the ethanolic extract of *P. ost*reatus fruit bodies steadily increased in direct proportion to increasing concentration of the extract. Reducing power of a 10 mg/ml concentration of mushroom extract was 1.367 and was relatively higher than that of BHT (1.192) (Jayakumar *et al.* 2009). Further, ethanolic extract from fruit bodies of *Pleurotus citrinopileatus* was reported to exhibit reducing power of 1.05 at 10 mg/ml (Lee *et al*. 2007).

Lipid peroxidation (LPO), a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids, inactivates cellular components and therein plays a key role in oxidative stress in biological systems (Niki 2010). Hence, inhibitory activity of mushroom extract on LPO was evaluated. Both mushroom extract and ascorbic acid standard inhibited lipid peroxidation (Table 4). At a concentration of 10 mg/ml, mushroom extract effected 51.2% inhibition of LPO activity and the ascorbic acid standard effected 92%. At least until now, there is no data available for comparison of our results obtained with FB-E extract on lipid peroxidation in the *in vitro* erythrocyte membrane model estimated by TBARS. Erythrocytes are excellent subjects for studies of biological effects of free radicals, since they are structurally simple, are continuously exposed to high oxygen tensions, the membrane lipids are composed partly of polyunsaturated fatty acid side chains which are vulnerable to peroxidation, and they have antioxidant enzyme systems (Konyalioglu *et al.* 2005).

In vitro antioxidant properties exhibited by *Pleurotus* mushroom preparation may be due to presence of antioxidant mycochemicals, like polyphenols. In addition, antioxidant activities (chelating ability of ferrous ion, inhibition of LPO and reducing power) found in polysaccharide extracts from widely used mushrooms *Ganoderma applanatum, Ganoderma lucidum*, *Lentinus edodes, Trametes versicolor* and *Pleurotus eryngii* does not negate the possible contribution of β-1,3-1,6-glucans polysaccharides to the antioxidant effect (Kozarski *et al.* 2012, Fu *et al.* 2016).

In our experiment of LPO inhibition, butylated hydroxyltoluene (BHT) was used as a standard. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are amongst the most commonly used synthetic antioxidants that are used in fats and oily foods to prevent oxidative deterioration. However, BHA and BHT have restricted use in foods as they are suspected carcinogens and may cause liver damage (Botterweck *et al.* 2000). This may explain why there is currently much research on the application of antioxidants from natural products. Although BHT and ascorbic acid, substances used as standards in our work, have significant antioxidant activity, they are additives and are used or are present in mg levels in foods. *Pleurotus* powder could be used in gram levels as functional food or nutraceutical, thus providing health protection to help humans reduce oxidative damage daily.

Research carried out this way, showed that polysaccharides of *P. tuber-regium* (Fr.) Sing. had strong antioxidant potency and might be exploited as effective natural antioxidant to alleviate oxidative stress. The antioxidant activities of two homogeneous polysaccharides, water-extracted polysaccharide (W-PTR) and alkali-extracted polysaccharide (A-PTR) were evaluated. Results indicated that W-PTR was stronger than A-PTR in superoxide scavenging activity, while A-PTR was stronger than W-PTR in the scavenging activities to hydroxyl, DPPH$^{\bullet}$, inhibition effects on liver lipid peroxidation, liver mitochondria swelling, and red blood cell (RBC) hemolysis (Wu *et al.* 2014).

In the link between antioxidant and antitumor activities, Ren *et al.* (2015) isolated the polysaccharide (PAP) from fruit bodies of *P. abalonus* and evaluated their antiproliferative activity in human colorectal carcinoma LoVo cells. PAP exerted a high antioxidant activity *in vitro* and a dose-dependent antiproliferative effect against LoVo cancer cells. Flow cytometry analysis demonstrated that PAP exhibited a stimulatory effect on apoptosis of LoVo cells and induced cell-cycle arrest at the S phase. PAP also increased generation of intracellular Radical Oxygen Species (ROS), critical mediators in PAP-induced cell growth inhibition. These findings suggested that PAP may serve as a potential novel dietary agent for human colon cancer chemoprevention.

Additionally, *Pleurotus eryngii* residue polysaccharides obtained by ultrafiltration showed *in vitro* antioxidant properties and cytotoxicities with CPPS-1 the strongest activity. Expression of tumor suppressor p53 and apoptosis activator Bax were up-regulated by CPPS-1 fraction while the expression of Bcl-2 was down-regulated. The results suggested that antitumor activity of CPPS-1 may be related to its capability of inducing apoptosis via activation of mitochondria apoptosis pathway (Ma *et al.* 2016).

CONCLUSIONS

Taken together, these observations indicate that *Pleurotus* sp. extracts (Myc-E and FB-E) and powders (FB-P) possess bioactivities involved in antitumor mechanism including immunostimulatory, antiproliferative and antioxidant toward free radicals. Both fruit bodies and mycelial preparations were well tolerated and can be used in formulation of consistent and safe mushroom products. Thus, *Pleurotus* sp., a common edible and medicinal mushroom, exerted health promoting benefits to maintain good health by activating the immune system for a multitude of defensive functions. *Pleurotus* sp. may be developed as a functional food and potential myco-therapeutic agent for human diseases, especially for enhancing

anticancer and immune responses. It appears that more studies are necessary to explore the complete structural characteristics of preparations tested, structure–activity relationship and molecular signaling pathways of their antitumor activity. As a result, future research may be oriented in that direction.

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14. CULTIVATION BIOTECHNOLOGY FOR *Volvariella* spp. IN MEXICO: ADVANCES, CHALLENGES AND PERSPECTIVES

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ABSTRACT

Edible fungi are a traditional food that has been eaten in Mexico since precolonial times and are currently an emerging agrofood industry supported mainly by production and commercialization of species of temperate climates. Considering the extensive biological and cultural diversity of the country, as well as the notable area of the country that has warm weather throughout most of the year, it is desirable to diversify with other species of commercial interest that can be grown under these conditions. Among fungi currently cultivated worldwide, *Volvariella* spp. are a productive alternative for tropical and subtropical regions, ideal for growing in rural areas since they only require a relatively small investment. Even though species such as *V. volvacea* and *V. bombycina* are collected and eaten by people in various regions of the country, development of biotechnology for their cultivation in Mexico has been slow and limited to laboratory experiments, the storage of genetic material and field trials to adapt their growth and development on different lignocellulose materials. This study summarizes results obtained to date and provides a prospective of the potential for producing these species at the regional level, based on existing genetic, agricultural and environmental resources.

Keywords: edible mushrooms, tropical species, paddy straw mushroom, cultivation prospects

INTRODUCTION

Mexico is one of the most important cultural and biological reservoirs of edible wild mushrooms in the world. This is mainly the result of the peculiar biological diversity, geological and geographic conditions, as well as the ancestral cultural history of the country (Pérez-Moreno *et al.* 2010) that have survived to the present day through texts, archeological relicts and traditional knowledge handed down from generation to generation (Garibay-Orijel *et al*. 2010). According to Boa (2004), in Mexico at least 303 species of fungi are used traditionally, the majority for culinary purposes.

In contrast, cultivation of edible fungi in Mexico dates back to only the 1930s and the first fifty years were focused on producing the white button mushroom, *Agaricus bisporus* (J.E. Lange) Imbach. It was only starting in the 1980s, in parallel with the consolidation of the mushroom industry in temperate-cold regions, that experimentation with species in tropical regions was presented as a novel feasible alternative, based on the fact that most of the commercial species grew wild in the country, especially those of the genera *Pleurotus* (Fr.) P. Kumm. and *Volvariella* Speg. (Guzmán *et al.* 1993). Additionally, academic groups interested in the study of wild germplasm and the implementation of techniques for growing commercially known species were formed (Martínez-Carrera 2000, Mata *et al.* 2016, Sánchez *et al.* 2016). Early studies focused on temperate species in the genus *Pleurotus*, but continued with species for which reproduction was potentially viable in tropical and subtropical regions of the country, such as *Auricularia* (Bull.) spp., *Cookeina sulcipes* (Berk.) Kunt., *Pleurotus djamor* (Rumph. ex Fr.) Boedijn and *Volvariella* spp. (Guzmán *et al.* 1993, Sánchez Vázquez *et al.* 1995a, 1995b).

Several edible species belonging to the genus *Volvariella* are grown commercially in southeastern Asia (Miles & Chang 2004). *Volvariella volvacea* (Bull.) Sing. is one of the most extensively cultivated mushrooms in tropical and sub-tropical regions and requires relatively high temperatures (28–35 °C) for vegetative growth and fruiting (Chen *et al.* 2003). It is widely used in traditional oriental cuisine and is

reported to possess anti-tumor, immunosuppressant and immunomodulatory effects (Buswell & Chen 2005, Mathew *et al*. 2008). It is known as a rich source of protein, fiber (chitin), vitamins (large amount of vitamin C, and also all water-soluble vitamins including riboflavin, biotin and thiamine), fat (5.7%), carbohydrates (56.8%), amino acids (all essential amino acids: alanine, arginine, glycine, serine etc.), unsaturated fatty acids, essential minerals (potassium, sodium and phosphorus) and has low calorific values (Chang 1980a, Ahlawat *et al*. 2016). The innumerable medicinal properties of *V. volvacea* along with its phytochemical properties are evidence of its value as a medicinal mushroom (Mathew *et al*. 2008). Annual production of *V. volvacea* has increased in recent years due to a higher demand for health foods. In 2010, Chinese production of *Volvariella* was 330,000 tons, accounting for 80% of the total world production of this genus. Although *V. volvacea* has been cultivated for 300 years, multiple problems associated with the practice has greatly restricted development of the industry (Bao *et al*. 2013).

Volvariella mushrooms are also known as "paddy straw or warm mushrooms". They are fast growing and under favorable production conditions, the total crop cycle is completed within 4-5 weeks. This mushroom can use a wide range of cellulosic materials and the C:N ratio needed is 40 to 60, quite high in comparison to other cultivated mushrooms (Biswas 2014). It can be grown quite quickly on non-composted substrates such as paddy straw and cotton waste (Miles & Chang 2004, Ahlawat 2011). In addition to these substrates, *V. volvacea* grows on water hyacinth, palm oil bunch wastes, pericarp wastes, banana leaves, and cotton waste (Chang 1980b, Belewu & Belewu 2005). Various grains (wheat, sorghum) and raw substrates (sawdust, rice bran, paddy husk etc.) have been used to produce spawn of *Volvariella* species (Chang 1978).

Paddy straw mushrooms have been traditionally cultivated on beds in the open field or using wooden frames (Chang 1993). In some regions, the bed is made of non-composted and unpasteurized bundles of rice straw exposed directly to the sun and covered with plastic sheets (Reyes 2000). The yield on beds is unstable and irregular, because the mycelium is exposed to microbial contamination and fluctuating environmental conditions. In recent times, development of indoor cultivation in growing houses under a controlled environment incorporated composting and pasteurization of substrates. Additionally, use of cotton waste in place of rice straw resulted in a significant increase of biological efficiency and more stable production but, it is still lower than other cultivated species (Kurtzman & Yung 1982, Chang 1993).

Volvariella basidiomes are commercially classified based on their stage of development: 1) primordia, 2) button, and 3) adult (Chang & Yau 1971). Each stage has different morphological and anatomical characteristics, with the button phase the most highly valued in the market since adult specimens deteriorate rapidly owing to polyphenol oxidative activity (Cho *et al.* 1982).

GENERAL CHARACTERISTICS OF *Volvariella* spp. AND THEIR DISTRIBUTION IN MEXICO

Volvariella Speg. is a cosmopolitan genus belonging to the *Pluteaceae* family in the Agaricales order and Basidiomycotina class (Singer 1986). It is characterized by basidiocarps initially covered with a veil that breaks during development, leaving as a remnant a membranous saccate volva at the base of the stipe. The spore print is pink to brownish pink. The context ("meat") is spongy, white to whitish, with a pleasant mushroom smell. At the microscopic level, it has tetrasporic basidia, spores ellipsoid, rather thick-walled, pinkish, cheilocystidia and pleurocystidia present, hymenophoral trama inverse (Li 1982, Seok *et al.* 2002).

Traditionally, the sexual pattern of mushrooms in the genus *Volvariella* has been described as primary homothallic, so the homokaryotic mycelium that emerges from the germination of a basidiospore is able to convert to the dikaryotic form and complete the sexual cycle without crossing (Chang & Yau 1971, Chiu *et al.* 1995). However, phylogenetic data presented by Bao *et al*. (2013) supported the notion that *V. volvacea*, like *A. bisporus*, is a pseudo-homothallic species. The fungi present multinucleate hyphae and

the dikaryotic mycelia have no clamp connections (Chang & Ling 1970). Recent studies on the size of *V. volvacea*'s genome indicate values of 35.7 mB and are very similar to those reported for *P. ostreatus* (Bao *et al*. 2013).

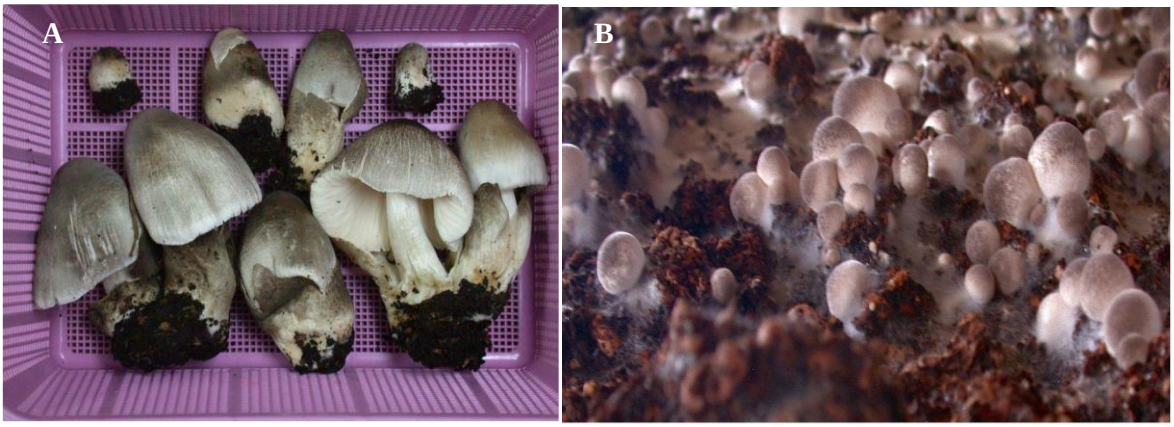

Figure 1. Basidiomes of *V. volvacea* collected from the wild on coffee pulp (A) and cultivated on mushroom beds at a Chinese farm (B).

In Mexico, 13 species of *Volvariella* have been cited, the majority of which are considered edible, with *V. volvacea* and *V. bombycina* (Schaeff.) Sing. the best known by rural inhabitants. These species grow wild on a variety of decomposing lignocellulose substrates: wood, bagasse (sugar cane, henequen from agave, tequila maguey), banana pseudostem and coffee berry pulp (Salmones *et al*. 1996) (Figure 1). *V. volvacea* is popularly known as the "bagasse mushroom", "sparrow-hawk's breast" or "pink mushroom", among other names, while *V. bombycina* var. *bombycina* is known as "banana mushroom" or "orchard fungus" and *V. bombicina* var. *flaviceps* is known as "yellow mushroom" or "little chicken" (Guzmán *et al*. 1993). These species have been cited for the states of Baja California, Jalisco, Morelos, Oaxaca, Puebla, Sonora, Quintana Roo, Yucatán and Veracruz (Ayala *et al.* 2015, Sobal Cruz *et al*. 2016, Pérez-Silva *et al*. 2006, Salmones *et al*. 1988, Vázquez *et al*. 1989).

EXPERIMENTAL WORK WITH *VOLVARIELLA* IN MEXICO

Studies carried out in Mexico to date have focused on establishing conditions required for its cultivation *in vitro*, with minimal *in situ* testing. Trials have focused on evaluating mycelial growth on agroindustrial residues available in the country, such as: grass straw, crop stubble, corn cobs, wood shavings from cazahuate *(Ipomoea arborescens)*, coconut fiber, coffee pulp, pineapple crown bracts, banana leaves and henequen bagasse (Acosta-Urdapilleta *et al.* 1992; Guzmán *et al*. 1993, Ancona & Salmones 1996, Salmones *et al.* 1996).

In the laboratory, Martínez *et al.* (1986) grew wild Mexican and commercial Asian strains of *V. volvacea* in culture medium in order to compare their macroscopic morphology and the microscopic characteristics of their mycelia. Later, Vela and Martínez-Carrera (1989) isolated monospore cultures of the Mexican and Asian strains and obtained fruit bodies on barley straw (Table 1), leading them to the conclusion that this American species, formerly known as *V. bakeri*, was a synonym of the Asian *V. volvacea*, the latter being the taxonomically correct name. In parallel, Salmones *et al*. (1988) compared mycelial growth of strains of *V. volvacea* and *V. bombycina* (Schaeffer: Fr.) Sing. on various culture media and lignocellulose materials, identifying suitable conditions for growing them *in vitro* and successfully developing *V. bombycina* basidiomes on oat straw.

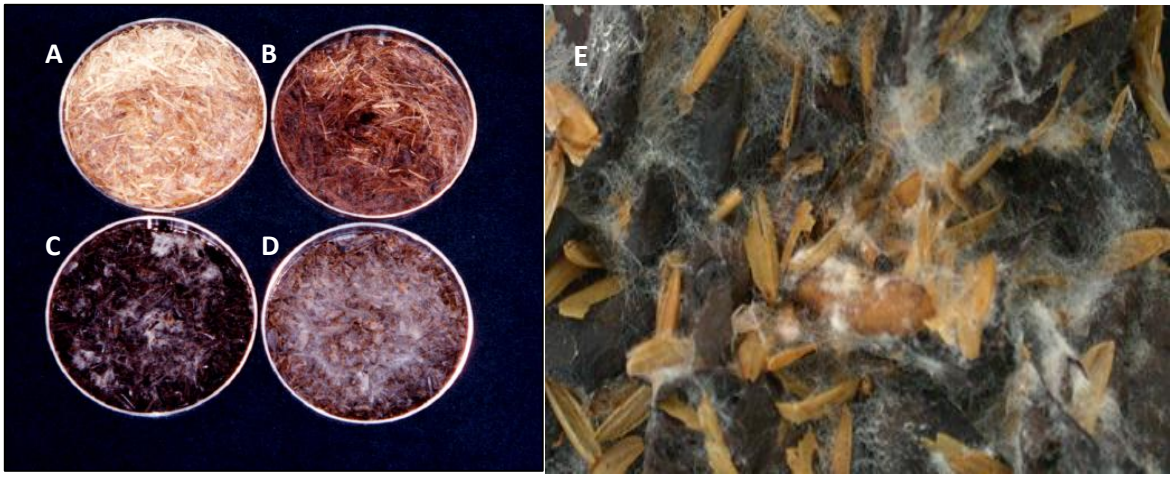

Figure 2. *Volvariella* mycelia growing on agro-industrial residues in the tropics. A: wheat straw, B: barley straw, C: banana leaves, D: rice straw, E: coffee pulp with rice husk.

Table 1. Biological efficiency of strains of *Volvariella volvacea* on various substrates made from raw materials found in Mexico.

Substrate	Biological efficiency (%)	Reference
Barley straw	5.3-22.7	Vela & Martínez Carrera (1989)
Barley straw	14.2	Salmones & Guzmán (1994)
Henequen bagasse	10.2	Ancona & Salmones (1996)
Pineapple bracts, coffee pulp, rice straw	6.2-33.8	Salmones *et al*. (1996)
Straw, banana leaves and pseudostem	19-25.6	Julián Carlos & Salmones (2006)
Barley straw, sugar cane bagasse Barley straw + 10 mM acetylsalicylic acid Sugar cane bagasse + 100 µM acetylsalicylic acid	46.4-64.8 27.1-135 25.5-34.2	Sobal Cruz *et al.* (2016)*

*Study done with *V. bombycina.*

Volvariella spp. are highly sensitive and will not fruit at low temperatures, so Salmones and Guzmán (1994) designed a laboratory fruiting chamber to maintain a suitable temperature range for development of basidiomes. Using this technique, Salmones *et al.* (1996) fruited wild strains of *V. volvacea* on agroindustrial residues as follows: coffee pulp, pineapple crown bracts and coconut fiber (Figure 2) and obtained biological efficiency values of 6.2 to 33.8%. Recently, Sobal Cruz *et al.* (2016) grew *V. bombycina* on barley straw and sugar cane bagasse, achieving a remarkable increase in productivity by applying acetylsalicylic acid in concentrations of 100 µM and 10 mM (Table 1).

At the *ex situ* level, the only study is that of Julián Carlos and Salmones (2006), who grew *V. volvacea* under rustic conditions in a banana-growing region in eastern Mexico (Veracruz state). They used waste from a banana harvest as the substrate (leaves and pseudostem) with biological efficiency values of 19 to 25.6 % (Table 1).

CONSERVATION OF WILD GERMPLASM

In Mexico, there are around eight fungi collections specializing in conserving species of alimentary and medicinal interest (Salmones & Mata 2012). Of these, Instituto de Ecología, A.C. (INECOL), Colegio de la Frontera Sur (ECOSUR) and Colegio de Postgraduados, *Campus* Puebla (COLPOS) curate isolates of *Volvariella* though there are no data on the total number or origin of the strains in these collections (Mata *et al*. 2016, Sánchez *et al.* 2016, Sobal Cruz *et al*. 2016).

The traditional method of conservation is by continuous sub-culturing on culture media or using sterilized water (Jong 1978). The germplasm of *Volvariella* is characterized by rapid mycelial growth. Routine storage at low temperatures (4 °C) causes mycelia to undergo autolysis. Low temperatures also cause fruit bodies to become soft, liquid, and even rotten (Chang 1978), which is why they should not be refrigerated. These two characteristics—fast growth and sensitivity to low temperatures—have adverse effects on conservation of strains, since periodical sub-culturing can induce physical and genetic changes that increase the risk of losing the biological material. Conservation of strains under ultracold conditions (-196 °C) is an advantageous alternative method since decreasing metabolism of the mushroom mycelium means it can be stored for extended periods of time (years) and, consequently decreases the possibility of physiological and genetic changes.

The Fungus Ceparium at the INECOL is one of the few institutes that has experimented with this conservation technique for edible species and has managed to implement a technique that uses sterilized grass seeds as a support for the mycelium (Mata *et al.* 2004). Following this methodology, Pérez and Salmones (1997) carried out trials to determine freezing protocols for the genetic material of 11 strains of *Volvariella*, evaluating different times of being in contact with a cryoprotectant solution of dimethyl sulfoxide (5% v/v). The recovery rate of strains was variable depending on conditions used. In general, 60 and 90 minutes of contact with the solution were the best for achieving total recovery of samples in the least amount of time (Figure 3).

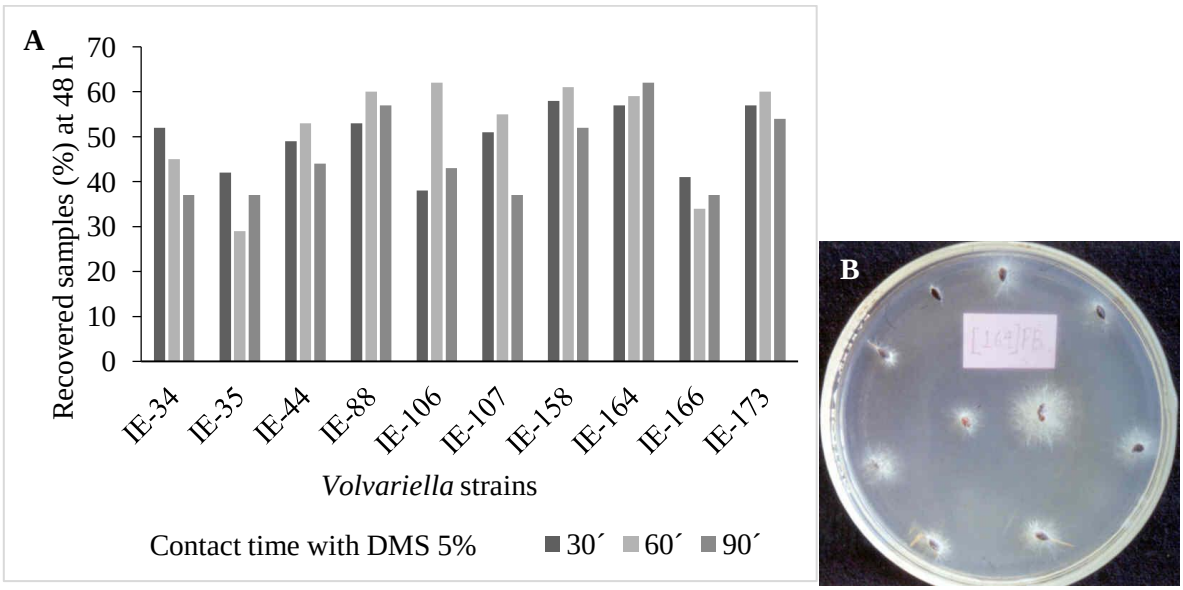

Figure 3. A: behavior of *Volvariella* strains during cryogenic storage. B: mycelia recovered after being frozen at -196 °C.

INOCULUM SUPPLEMENTATION TRIALS

Preparation of inoculum or spawn is the basis of commercial mushroom cultivation. For *Volvariella,* various lignocellulose materials are used commercially, either alone or in combination to prepare the inoculum. Best results were obtained with cotton, coconut palm and tea crop waste, as well as rice straw (Miles & Chang 2004). In Mexico, owing to its greater availability, grass straw was initially used as the substrate to prepare inoculum (Guzmán *et al*. 1993). The use of this organic material, however, ideally requires prior aerobic fermentation that increases the availability of the cellulose fraction. This is because the enzyme system of *Volvariella* is poorly equipped to degrade the ligninolytic fraction (Cai *et al.* 1999). Therefore, it necessary to have additional physical space for substrate fermentation.

One alternative for preparing inoculum of *Volvariella* is to use grass seeds (Quimio 2002). For this reason, in our laboratory, we have evaluated various formulations using wheat, millet and sorghum grains alone or mixed with carbohydrate-rich supplements (corn flour and coffee pulp) and limestone ($CaCO_3$) (Julián Carlos 2006). The results did not indicate a specific preference by the strains for any particular grain or supplement, although some combinations such as millet with corn flour 90:10 (p/p) and sorghum with limestone (99.5:0.5) (p/p) favored mycelial growth of *Volvariella*, which covered 59.52 cm^2 (93%) and 50.12 cm^2 (78%) of the Petri dishes' surface after 6 days of incubation (Table 2).

Table 2. Development of *V. volvacea* mycelium in formulations evaluated after six days of incubation.

Formulation*		Area (cm^2)	Petri dish area covered (%)	pH
Wheat	NS	9.95	15.6	6
	CF	22.84	35.9	5.8
	CP	12.78	20.1	5.6
	L	24.6	38.7	7.9
Millet	NS	38.35	60.3	6
	CF	59.52	93.6	5.6
	CP	30.49	47.9	5.6
	L	3.4	5.34	8.2
Sorghum	NS	39.45	62.0	6
	CF	34.14	53.7	5.9
	CP	27.16	42.7	5.8
	L	50.1	78.8	8.2

*NS: seeds with no supplementation, CF: seeds with corn flour (90:10), CP: with coffee pulp (90:10) and L: with limestone, $CaCO_3$ (99.5:0.5).

PERSPECTIVES

It is evident that mushroom diversity in the tropics is abundant and that humanity has yet to study or take advantage of the vast majority of the species, many of which will probably go extinct before becoming known to science. In Mexico alone, it is estimated that we have studied a mere 4.5% of our mycological diversity (Guzmán 1998).

Professor S. T. Chang, a renowned specialist in the cultivation of tropical mushrooms, thinks that the tropical regions of the world are suitable for developing this biotechnology, mainly because they have the following characteristics: a) wet, warm climate and an abundant supply of agricultural waste, b) mushrooms are relatively fast-growing organisms; some tropical mushrooms can be harvested and consumed within 10 days after spawning, c) mushroom cultivation is labor intensive, however, this may not be a problem in tropical regions, d) while land availability is usually a limiting factor in most types of primary production, cultivating mushrooms requires little space because they can be stacked using trays, and e) mushrooms have been accepted as human food from time immemorial and can immediately supply additional protein (Chang 2007).

For the specific case of growing *Volvariella* in Mexico, the country satisfactorily meets the conditions listed below: a) close to 28% of its area has a warm climate (Villers-Ruiz & Trejo-Vázquez 1998) as recommended for the *in situ* reproduction of this genus, b) annually, more than 60 billion tons of organic waste material are produced during harvest and post-harvest management of at least 20 different agricultural crops (Valdez-Vázquez *et al*. 2010), and the chemical composition and physical structure of most of these materials is suitable for mushroom production. An example of this is sugar cane, one of the main agricultural crops in Mexico, with around 570,000 hectares designated to its cultivation. Depending

on the harvest method used, sugar cane cultivation produces 28 to 57 million tons of organic material that is left in the fields (CONADESUCA 2016). Using some of this waste for growing mushrooms would represent a sustainable way of using it that benefits the environment, c) wild strains of *Volvariella* have fruited 13 days after spawning (Salmones *et al.* 1996) and d) rural inhabitants appreciate and eat both *V. volvacea* and *V. bombycina* (Guzmán *et al.* 1993).

In Mexico, there are academic groups in research centers and universities located in tropical and subtropical regions that specialize in mushroom cultivation. These groups could have a very important impact by generating knowledge about techniques, substrates, varieties, control of diseases, etc., applicable to wild species. They also have the capacity to disseminate knowledge, through training courses and by providing advice to growers, both from Mexico and other countries on the continent interested in working with tropical species (Martínez-Carrera *et al.* 2016). Furthermore, some of these institutions have experience in the preservation, reproduction and genetic improvement of wild germplasm (Mata *et al*. 2016, Sánchez *et al.* 2016, Sobal Cruz *et al*. 2016).

Moreover, the national production of edible and medicinal mushrooms is noteworthy and accounts for about 50% of Latin America's entire production. It is considered the most important emerging agrofood chain in the country, with a direct impact on food production for direct human consumption and on producing new, high added value products with functional and medicinal properties (Martínez-Carrera *et al*. 2016). Added to this consolidated production chain and the expansion in national consumption, the strategic geographic location of Mexico has favored its leadership in this agroindustry, with a sustained increase in exports to Latin American countries (INEGI 2016), some of which, like Peru and Guatemala, share the ancestral tradition of eating mushrooms (Garibay-Orijel *et al*. 2010, Pavlich 2001). In Guatemala, there have even been trials with the aim of cultivating *Volvariella* (De León *et al.* 1987).

In spite of notable growth in the agroindustry dedicated to cultivating edible mushrooms in Mexico, much remains to be done in the areas of species diversification and innovation in growing techniques. Specifically, to establish commercial cultivation of *Volvariella*, it is necessary to deepen our understanding of the following aspects: a) basidiome production in greenhouse or field conditions, in order to develop a systematic production system, b) an evaluation of production of wild germplasm, and c) how to improve productivity of strains on residues available in the country, all in order to make the process more economical.

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15. A *Pleurotus* spp. HYDROALCOHOLIC FRACTION POSSESS A POTENT *in vitro* OVICIDAL ACTIVITY AGAINST THE SHEEP PARASITIC NEMATODE *Haemonchus contortus*

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ABSTRACT

Gastrointestinal parasitic nematodes cause important economic harm to animal health, seriously affecting the livestock industry. Worldwide, economic losses from nematodiasis is reported at more than USD 10 billion a year in anthelmintic treatments alone in 2013. In Mexico, the livestock industry has reported losses of about $8.902 million Mexican pesos in the zootechnical potential of ruminants that interfere in the profitability of livestock farms. So far, these diseases have been controlled by use of anthelmintic products (AH). In addition, improper use of AH contributes to the imbalance of the environment, as well as an ecotoxicological risk to soil, plants, aquifers and beneficial organisms. In this context, the search for alternative and sustainable complementary methods that reduce the need for use of synthetic products of chemical origin, for example natural derivatives of edible fungi with a nematicidal effect, is evident. The edible fungi *Pleurotus* spp. possesses nutraceutical and therapeutic properties including their use as anti-parasitic. In the present investigation, *in vitro* effects of compounds derived from *Pleurotus* spp. were assessed against various stages of the parasitic nematode *Haemonchus contortus*. *Pleurotus ostreatus* had higher nematicidal activity than *P. eryngii*. The "F" fraction of hydroalcoholic extract of *P. ostreatus* mycelium showed the highest nematicidal activity (25.29%) with respect to other fractions. FpMeOH and FMeOH fractions had the highest L_4 mortality rate of 84.8 and 100, respectively at 24 and 72 h. The spent mushroom substrates (SMS) of *P. djamor* had biological activity against *H. contortus* in its egg stages and L_3. Furthermore, such activity is possibly influenced by the composition and/or the type of substrate. EHA-SA11 was lethal against L_3 exsheathed with a 45% mortality at 20 mg/ml at 72 h postexposure, but did not affect nematode eggs. In contrast, EHA-SA13 was the most effective against parasite eggs with 100% inhibition starting at 2.5 mg/ml that was determined at CL_{90}= 625 μg/ml. The use of a "nutraceutical" food based on SMS of edible mushroom culture represents a potential method of prevention of nematode infections.

Keywords: ovine cattle, nematicidal mushrooms, biological control

INTRODUCTION

Gastrointestinal nematodes (GIN) are a major concern for livestock breeders worldwide, as they cause anemia, diarrhea, weight loss and even death in young animals (von Son de Fernex *et al*. 2014). Among various gastrointestinal parasitic nematodes species, *H. contortus*, possess haematophagia properties (Besier *et al*. 2016a,b). This parasite has been found with a high prevalence in different parts of the world with an enormous global economic impact. Treatment costs against parasites alone are more than USD 10 billion a year and do not include productivity losses (Roeber *et al*. 2013). In Mexico, losses of $8,902 million pesos have been reported in the zootechnical potential of ruminants, interfering with the profitability of livestock farms (Rodríguez-Vivas *et al*. 2017). Nematode parasitosis of cattle and small

ruminants has been treated for decades with chemical anthelmintic products that possess undesirable effects; for example, their excessive and continuous use has led to the development of a severe problem of resistance in the parasites to these compounds. Additionally, chemical anthelmintic drugs affect beneficial organisms such as dung beetles *ie., Onthophagus landolti* and *Canthon indigaceus chevrolati* (Basto-Estrella *et al*. 2014). Therefore, it is necessary to seek alternatives to implement sustainable control methods of these parasitic diseases. Edible fungi are considered a traditional component in the diets of various communities nationally and globally, with important nutritional characteristics. These edible fungi have not only been appreciated as food but have served as an important utility within traditional medicine. They contain compounds with different therapeutic properties, such as anti-inflammatory, antihypertensive, immuno-modulators, anti-viral, anti-microbial, anti-carcinogenic, anti-oxidant, anti-cholesterolemic, anti-allergic, insecticidal, anti-fungal and anti-parasitic (Glamočlija *et al.* 2015). Species of the edible fungus *Pleurotus* possess nutraceutical and nematicidal properties (Khaund and Joshi 2015, Kirsch *et al*. 2016). Regarding nematicidal properties, 23 species of the genus *Pleurotus* are active against nematodes (Li and Zhang 2014). In addition, some products such as proteases (André-Genier *et al*. 2015), fatty acids (Pineda-Alegría *et al*. 2017), alkaloids, peptidic compounds, terpenes (Li *et al*. 2007), condensed tannins and phenolic compounds (Ganeshpurkar *et al*. 2012) obtained from *Pleurotus* spp. have shown antiparasitic activity (Shariat *et al*. 1994, Palizi *et al*. 2009, Del Carmen *et al*. 2015). Secondary metabolites produced by *Pleurotus* species have nutraceutical characteristics and have been mostly isolated from basidiomes of the fungus. In the present investigation, products derived from *Pleurotus* spp. were tested *in vitro* against different stages of the sheep parasitic nematode, *H. contortus*.

MATERIALS AND METHODS

Biological material

Mushrooms

Mycological material (mycelium, basidiomes and spent mushroom substrate) was provided by the Tropical Mushrooms Laboratory of the Southern Border College (ECOSUR), Tapachula Headquarters, Chiapas, Mexico.

Mycelium

Mycelium of *Pleurotus ostreatus* ECS-1123 and *P. eryngii* ECS-1292 were used and mycelium was produced on an agar medium of whole wheat flour (HIT) contained in Petri dishes after 14 days' growth (Comans-Pérez *et al.* 2014).

Basidiomes

Basidiomes of *P. djamor* strain ECS-123 were produced following methodology of Avendaño and Sánchez (2013).

Pleurotus djamor production substrate

Various ingredients were used to prepare substrate mixtures to promote *P. djamor* production. The composition of each substrate used is shown in Table 1.

Nematode

The *H. contortus* strain was obtained from the INIFAP "Las Margaritas" Experimental Site and maintained with periodic passes in experimental sheep at the Helminthology Unit of the CENID-Veterinary Parasitology in Jiutepec, Morelos, Mexico (Campos *et al.* 1990).

Table 1. Substrates for *P. djamor* production

Substrate	Mixture 1 (g)	Mixture 2 (g)	Mixture 3 (g)	Mixture 4 (g)
06	12-20	60-71	11-18	18-25
10	30-35	0	30-40	43-50
11	30-40	30-40	0	40-50
12	40-48	38-45	38-45	0
13	28-38	27-35	27-37	40-45

Substrates were weighed to obtain a similar starting amount in dry weight at 100 g.

Eggs and L_3 larvae of *H. contortus*

One three-month-old male sheep that was previously infected with 350 third stage larvae (L_3) per kg/live weight was used. After the pre-patent period (21 days), samples of fresh feces collected directly from the rectum of the donor ovine were taken to confirm infection using the McMaster technique. Feces were macerated with potable water until a homogeneous mixture was obtained and filtered through various sieves of descending sizes. Eggs were recovered and purified by a gradient with 40% sucrose and centrifugation at 3500 rpm for 5 min. The ring formed by the eggs was recovered and washed using sterile distilled water, by centrifugation. For the production of L_3 larvae, samples of feces from the sheep were taken after the pre-patent period and a coproculture was made in a plastic container to obtain L_3. Finally, at 7 days, L_3 larvae were recovered using the Baermann technique and stored in a refrigerator at 4 °C until used (Liébano *et al*. 2011).

Exsheathed of *H. contortus* L_3 larvae

Three ml of 6% sodium hypochlorite were used to make dilutions and obtain concentrations of 3, 1.5, 0.750, 0.375, and 0.187%. This process required 7 min, then three washes were performed with distilled water and centrifuged at 2500 rpm for 1 min each wash; the supernatant was discarded and the larvae were recovered (Liébano *et al*. 2011).

Development of *H. contortus* L_4 larvae

The fourth evolutionary stage was obtained under a controlled CO_2 atmosphere, using L_3 exsheathed following methodology described by Ramírez *et al.* (2006).

Preparation of hydroalcoholic extracts of mycelium *P. ostreatus* and *P. eryngii*

A hydroalcoholic solution was prepared using a 70:30 ethanol-water ratio. The solution covered all fungal material contained in an Erlenmeyer flask and was allowed to stand for 24 h. Subsequently, samples were subjected to the distillation process using a rotavaporator and finally, the concentrated extract was dried at high vacuum using a lyophilizer (Huicochea-Medina *et al.* 2015).

Chemical fractionation of a hydroalcoholic extract of the mycelium of *P. ostreatus*

A hydroalcoholic extract (3 g) of mycelium of *P. ostreatus* was weighed and adsorbed onto 7 g of normal phase silica and a glass column was packed with 60 g of normal phase silica (Durst and Gokel 2007). The elution was started with a dichloromethane system (100%) and 10% volume-to-volume increments of methanol were made, ending with a dichloromethane-methanol system (50:50 v/v) and ending with methanol (100%) washing (Huicochea-Medina *et al.* 2015).

Purification of fraction "F" from *P. ostreatus* mycelium

Purification was performed by high performance liquid chromatography (HPLC). Fraction "F" extract (3 mg) was weighed and diluted in 1 ml of methanol HPLC to have a concentration of 3 mg/ml. From this solution, 1 ml was taken and the sample injected into the chromatograph. Previously, equipment was washed with water and methanol HPLC grade. Samples were programmed for detection at a wavelength of 280 nm (González 2010).

Chemical fractionation of fruit bodies of *P. djamor*

Fruit bodies were fragmented and placed in a 40:60 water-ethanol solution. The solution covered the fungal material in a 3:1 ratio and was allowed to soak for 24 h at room temperature. Subsequently, the sample (hydroalcoholic extract) was filtered with a gauze and cotton system, concentrated in a rotary evaporator and dried under high vacuum using a freeze-dryer. Dry material was fractionated by the open column chromatography technique using dichloromethane-methanol systems. Fractions evaluated were obtained in a 1:1 dichloromethane-methanol system. Two fractions were recovered: one-part from the sediment and another part remained soluble. They were denominated "FpMeOH" and "FMeOH".

Obtaining hydroalcoholic extracts from spent mushroom substrate

The dry weight of each of five spent mushroom substrates (SMS) previously described in Table 1, were individually placed in 1 liter flasks containing a hydroalcoholic solution in a ratio of 70:30, ethanol:water. A volumetric ratio of 3:1, hydroalcoholic solution:SMS was used. Samples were macerated at room temperature for 24 h protected from light**.** Biomass was removed by filtration through sterile cotton gauze and each of the filtrates were collected in 1 liter flasks. Filtrates were concentrated by removal of solvent by vacuum distillation in a rotary evaporator at 49 °C temperature and 80 rpm. Extracts were obtained by lyophilization of the concentrated macerates. Five hydroalcoholic extracts of depleted substrate (EHA-SA) were obtained and were named as follows: EHA-SA06, EHA-SA10, EHA-SA11, EHA-SA12 and EHA-SA13.

Experimental designs

Bioassays were carried out in 96-well microtiter plates as described below:

In vitro* comparison of hydroalcoholic extracts and mycelial fractions of *P. ostreatus* and *P. eryngii* against L_3 of *H. contortus

In each well, 80 µl of hydroalcoholic extract of *P. ostreatus* and *P. eryngii* or fractions denominated: B, D, E, F and G of mycelium of *P. ostreatus* were placed at a concentration of 400, 200, 100, 50, 25 ml and 20 µl of the aqueous suspension containing 200 L_3 of *H. contortus* sheathed and exsheathed, leaving a final volume of 100 µl. Two control groups were included: positive control containing distilled water and a negative control with commercial ivermectin [10 mg/ml], with four replicates per treatment and three post-challenge readings: 24, 48, and 72 h. Data were analyzed using the SAS GLM procedure (SAS, 2004). Adjusted mortality was calculated according to the formula: % nematicidal effectiveness = average of the control-average of the treated group/average of the control group * 100 (Eguale and Giday 2009). Subsequently, mortality was transformed to a square root and then to a sinus arc to homogenize the variance and obtain an approximation to a normal distribution. A 2x5x3 factorial design was used where the main effects were: hydroalcoholic extracts obtained from *P. ostreatus* and *P. eryngii*, five concentrations (400, 200, 100, 50 and 25) and 3 times (24, 48 and 72 h). Duncan's test (Steel and Torrie 1988) was used to separate means according to the following model: $Yijkl = \mu + F_i + D_j + T_k + F * T_{ik} + D * T_{jk} + F * D_{ij} + D * Et * T_{ijk} + \varepsilon_{ijkl}$ Where: Y_{ijkl} = response variable (adjusted mortality). M = population

mean. F_i = fixed effect of extracts, D_j = fixed effect of j-th concentration (j = 400, 200, 100, 50 and 25). T_k = fixed effect of k-th reading time (k = 24, 48, 72). $(F * T)_{ik}$ = effect of extract and time. $(D * T)_{jk}$ = effect of the jth concentration and time. $F * D_{ij}$ = combined effect of extract and concentration. $F * D * T_{ijk}$ = effect of extract, concentration and time. ε_{ijkl} ~NI (0, 2e). A second 5x5x3 factorial design was used where the effects were: fractions obtained from *P. ostreatus* (B, D, E, F and G), 5 concentrations (400, 200, 100, 50 and 25) and 3 times (24, 48 and 72h), the same statistical model described above was used.

In vitro comparison of two methanolic fractions (FpMeOH and FMeOH) of basidiomes of *P. djamor* against L_4 larvae of *H. contortus*

100 L_4 and a final volume of 100 µl were placed in each well. Concentrations (via serial dilutions) for the fraction that precipitated (FpMeOH) were: 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40 and 60 mg/ml, whereas for the fraction that was soluble (FMeOH) were: 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 mg/ml. A negative control (enriched Hanks medium) and a positive control (Ivermectin 5 mg/ml) were used. Plates were then covered with foil and incubated at 28 ±1 °C in a CO_2 incubator. Readings were performed at 24, 48 and 72 h post-confrontation. For statistical analysis, a completely randomized design was used (Buzatti *et al*. 2015). An analysis of variance and a comparison of means by Tukey's method (α=0.05) were performed. SAS (2004) software was used. Adjusted mortality was calculated according to the formula: % nematicidal effectiveness = mean of the control group - mean of the treated group/mean of the control group * 100.

In vitro evaluation of five hydroalcoholic extracts of depleted substrates of *P. djamor* against eggs and L_3 larvae of *H. contortus*

A bioassay was performed for each of the five hydroalcoholic extracts from different *P. djamor*-SMS named: EHA-SA (06, 10, 11, 12 and 13) by placing six concentrations (0.5, 1.25, 2.5, 5, 10 and 20 mg/ml) dissolved in sterile distilled water and their respective controls, where 1% commercial ivermectin was used as a positive control in egg inhibition tests and mortality of *H. contortus* L_3 larvae. Four replicates (n=4) of each treatment were used by placing 50 µl of treatment at concentrations of: 1, 2.5, 5, 10, 20 and 40 mg/ml to obtain previously described working concentrations and 50 µl of an aqueous suspension containing 100 eggs and 200 L_3 of *H. contortus* sheathed. The final volume was 100 µl. Inoculated microplates were incubated at 29 °C and protected from light until they were read (48 and 72 h for eggs and 24, 48 and 72 h for L_3 sheathed) where aliquots were observed corresponding to each replicate of treatments in a 40X light field microscope. *H. contortus* eggs and/or larvae present in each of the treatments were quantified to obtain percent inhibition of hatching of each replicate as well as live larvae and dead larvae confirmed by physical stimulation. Subsequently, percentages of the four replicates were used to obtain percent inhibition and corrected mortality rate that was determined based on the Schneider-Orelli formula (1972): % inhibition of corrected eggs = group mean mean-control group / mean control group mean * 100. Data analysis was performed individually for each of the five EHA-SAs (06, 10, 11, 12 and 13) from the data weighted with the mean inhibition percentage of the corresponding negative control. We used a variance analysis of treatments in a completely randomized design and means were compared according to Tukey's test at a level of significance of 0.05. The statistical model used is as follows:

$$Y_{ij} = \mu + Extract_i + Error_j$$

Where:
Y_{ij}= Variable response (Inhibition at 48,72 h; mortality at 24, 48 y 72 h)
μ= Overall average
$Extract_i$= Effect of treatment (EHA-SA06, EHA-SA10, EHA-SA11, EHA-SA12, EHA-SA13)
$Error_j$= Random error

RESULTS

In vitro comparison of hydroalcoholic extracts and mycelial fractions of *P. ostreatus* and *P. eryngii* against L_3 of *H. contortus*

Results of nematicidal efficacy of the hydroalcoholic extract of *P. ostreatus* and *P. eryngii* against *H. contortus* L_3 sheathed at five different concentrations are presented in Table 2. Highest mortality rate of *P. ostreatus* was 23.3 at a concentration of 100 µg/ml at 72 h and for *P. eryngii* of 16.6% at 24 h at 200 µg/ml.

Table 2. *In vitro* interaction of *Haemonchus contortus* infective larvae with a hydroalcoholic extract of *Pleurotus ostreatus.*

Treatment	Concentration	Time (h)	X, SD	Mortality (%)
Pleurotus ostreatus ECS-1123	25 µg/ml	24	0.92 ± 0.38	11.78[a]
		48	1.20 ± 0.31	12.20[a]
		72	0.75 ± 0.23	13.51[a]
	50 µg/ml	24	0.98 ± 0.25	10.51[a]
		48	1.15 ± 0.42	11.59[a]
		72	1.07 ± 0.23	17.69[b]
	100 µg/ml	24	0.77 ± 0.41	8.96[a]
		48	1.15 ± 0.12	12.13[a]
		72	1.57 ± 0.42	23.33[b]
	200 µg/ml	24	0.95 ± 0.31	9.57[a]
		48	1.17 ± 0.57	10.70[a]
		72	1.17 ± 0.28	16.54[b]
	400 µg/ml	24	1.17 ± 0.20	11.3[a]
		48	1.07 ± 0.53	12.72[a]
		72	1.52 ± 0.63	21.28[b]
Controls	Distilled water	24	0.10 ± 0	1.28
		48	0.07 ± 0.05	0.64
		72	0.02 ± 0.05	0.45
	Ivermectin (10 mg/ml)	24	9.05 ± 0.93	100
		48	9.10 ± 1.49	100
		72	6.07 ± 0.71	100

X = Average; SD = Standard Deviation. Values followed by a different letter indicate statistical differences (P<0.05).

Results of nematicidal effectiveness of fractions against *H. contortus* L_3 sheathed are shown in Tables 3-7. Fraction "F" had the highest mortality rate of 25.2% at 72 h with a concentration of 400 µg/ml, while the lowest percentage was from fraction "D" with 1.64.

Table 3. *In vitro* interaction of infective larvae of *Haemonchus contortus* with the fraction "B" of the hydroalcoholic extract of the mycelium of *Pleurotus ostreatus*.

Treatment	Concentration	Time (h)	X, SD	Mortality (%)
Fraction B	25 µg/ml	24	0.47 ± 0.29	5.13[ab]
		48	0.58 ± 0.26	5.36[ab]
		72	0.52 ± 0.15	6.34[ab]

	50 µg/ml	24	0.58 ± 0.22	4.96[ab]
		48	0.50 ± 0.45	4.48[ab]
		72	0.42 ± 0.12	4.22[b]
	100 µg/ml	24	0.85 ± 0.43	8.19[a]
		48	0.47 ± 0.17	4.43[ab]
		72	0.75 ± 0.28	8.19[a]
	200 µg/ml	24	0.55 ± 0.17	6.94[ab]
		48	0.62 ± 0.28	6.02[ab]
		72	0.67 ± 0.33	6.63[ab]
	400 µg/ml	24	0.70 ± 0.21	5.49[ab]
		48	0.62 ± 0.12	5.68[ab]
		72	0.67 ± 0.27	7.54[ab]
Controls	Distilled water	24	0.05 ± 0.06	0.68
		48	0.02 ± 0.05	0.26
		72	0.05 ± 0.05	0.62
	Ivermectin (10 mg/ml)	24	9.48 ± 0.82	100
		48	10.65 ± 0.9	100
		72	8.02 ± 0.87	100

Average (X) and standard deviation (SD) were calculated from three repetitions each. Values followed by a different letter indicate statistical differences (P<0.05) among concentrations and time.

Table 4. *In vitro* interaction of infective larvae of *Haemonchus contortus* with fraction "D" of a hydroalcoholic extract of the mycelium of *Pleurotus ostreatus*.

Treatment	Concentration	Time (h)	X, SD	Mortality (%)
Fraction D	25 µg/ml	24	0.33 ± 0.13	2.68[b]
		48	0.50 ± 0.16	5.31[b]
		72	0.15 ± 0.13	3.59[b]
	50 µg/ml	24	0.20 ± 0.15	1.64[b]
		48	0.40 ± 0.08	3.5[b]
		72	0.25 ± 0.13	5.18[b]
	100 µg/ml	24	0.37 ± 0.28	2.88[b]
		48	0.62 ± 0.45	5.85[ab]
		72	0.12 ± 0.12	2.09[b]
	200 µg/ml	24	0.53 ± 0.15	4.17[b]
		48	1.32 ± 0.92	9.61[a]
		72	0.32 ± 0.17	3.97[b]
	400 µg/ml	24	0.80 ± 0.80	5.83[b]
		48	0.35 ± 0.19	3.53[b]
		72	0.30 ± 0.18	3.64[b]
Controls	Distilled water	24	0.02 ± 0.05	0.23
		48	0	0
		72	0	0
	Ivermectin (10 mg/ml)	24	15.6 ± 4.43	100
		48	12.2 ± 0.59	100
		72	10.42 ± 1.00	100

Average (X) and standard deviation (SD) were calculated from three repetitions each. Values followed by a different letter indicate statistical differences (P<0.05) among concentrations and time.

Table 5. *In vitro* interaction of infective larvae of *Haemonchus contortus* with the fraction "E" of a hydroalcoholic extract of the mycelium of *Pleurotus ostreatus.*

Treatment	Concentration	Time (h)	X, SD	Mortality (%)
Fraction E	25 µg/ml	24	0.30 ± 0.08	6.06^{c}
		48	0.45 ± 0.30	7.03b^{c}
		72	0.30 ± 0.18	6.09^{c}
	50 µg/ml	24	0.50 ± 0.52	6.82b^{c}
		48	0.63 ± 0.39	9.12b^{c}
		72	0.70 ± 0.11	10.04^{ab}
	100 µg/ml	24	0.72 ± 0.26	7.02^{bc}
		48	0.43 ± 0.26	5.33^{c}
		72	1.02 ± 0.28	13.09^{a}
	200 µg/ml	24	0.43 ± 0.17	4.67^{c}
		48	0.43 ± 0.28	5.28^{c}
		72	0.80 ± 0.62	7.74^{bc}
	400 µg/ml	24	0.75 ± 0.17	6.71^{bc}
		48	0.55 ± 0.19	5.80^{c}
		72	0.60 ± 0.27	6.52^{c}
Controls	Distilled water	24	0.05 ± 0.06	0.68
		48	0.02 ± 0.05	0.26
		72	0.05 ± 0.05	0.62
	Ivermectin (10 mg/ml)	24	9.48 ± 0.82	100
		48	10.65 ± 0.90	100
		72	8.02 ± 0.08	100

Average (X) and standard deviation (SD) were calculated from three repetitions each. Values followed by a different letter indicate statistical differences (P<0.05) among concentrations and time.

Table 6. *In vitro* interaction of infective larvae of *Haemonchus contortus* with fraction "F" of a hydroalcoholic extract of the mycelium of *Pleurotus ostreatus*

Treatment	Concentration	Time (h)	X, SD	Mortality (%)
Fraction F	25 µg/ml	24	1.62 ± 0.27	10.19^{cd}
		48	1.65 ± 0.05	10.1^{cd}
		72	2.15 ± 0.28	16.2^{b}
	50 µg/ml	24	1.55 ± 0.66	9.24^{cd}
		48	2.12 ± 0.39	11.89^{c}
		72	1.80 ± 0.40	14.12^{b}
	100 µg/ml	24	1.90 ± 0.70	10.97^{cd}
		48	2.82 ± 0.12	13.51^{bc}
		72	2.37 ± 0.54	16.73^{b}
	200 µg/ml	24	1.62 ± 0.35	11.02^{cd}
		48	2.55 ± 0.42	12.37^{c}
		72	1.97 ± 0.22	14.93^{b}
	400 µg/ml	24	1.10 ± 0.21	7.24^{d}
		48	2.92 ± 0.65	16.34^{b}
		72	3.27 ± 0.35	25.29^{a}
Controls	Distilled water	24	0.20 ± 0	1.25
		48	0.25 ± 0.24	1.65
		72	0.10 ± 0.08	0.74
	Ivermectin (10 mg/ml)	24	11.10 ± 1.73	100
		48	12.83 ± 3.20	100
		72	12.72 ± 1.04	100

Average (X) and standard deviation (SD) were calculated from three repetitions each. Values followed by a different letter indicate statistical differences (P<0.05) among concentrations and time.

Table 7. *In vitro* interaction of infective larvae of *Haemonchus contortus* with fraction "G" of a hydroalcoholic extract of mycelium of *Pleurotus ostreatus*

Treatment	Concentration	Time (h)	X, SD	Mortality (%)
Fraction G	25 µg/ml	24	1.28 ± 0.13	10.28[ab]
		48	1.65 ± 0.20	9.42[ab]
		72	1.00 ± 0.40	7.73[b]
	50 µg/ml	24	1.50 ± 0.26	10.90[ab]
		48	1.95 ± 0.33	11.90[ab]
		72	1.30 ± 0.21	9.50[ab]
	100 µg/ml	24	1.50 ± 0.39	9.42[ab]
		48	1.70 ± 0.54	9.97[ab]
		72	1.60 ± 0.21	9.22[ab]
	200 µg/ml	24	1.50 ± 0.26	11.10[ab]
		48	1.35 ± 0.34	8.01[b]
		72	1.50 ± 0.21	9.78[ab]
	400 µg/ml	24	1.47 ± 0.33	10.2[ab]
		48	1.63 ± 0.53	9.54[ab]
		72	1.75 ± 0.46	11.51[a]
Controls	Distilled water	24	0.20 ± 0	1.25
		48	0.25 ± 0.24	1.65
		72	0.10 ± 0.08	0.74
	Ivermectin (10 mg/ml)	24	11.10 ± 1.73	100
		48	12.83 ± 3.20	100
		72	12.72 ± 1.04	100

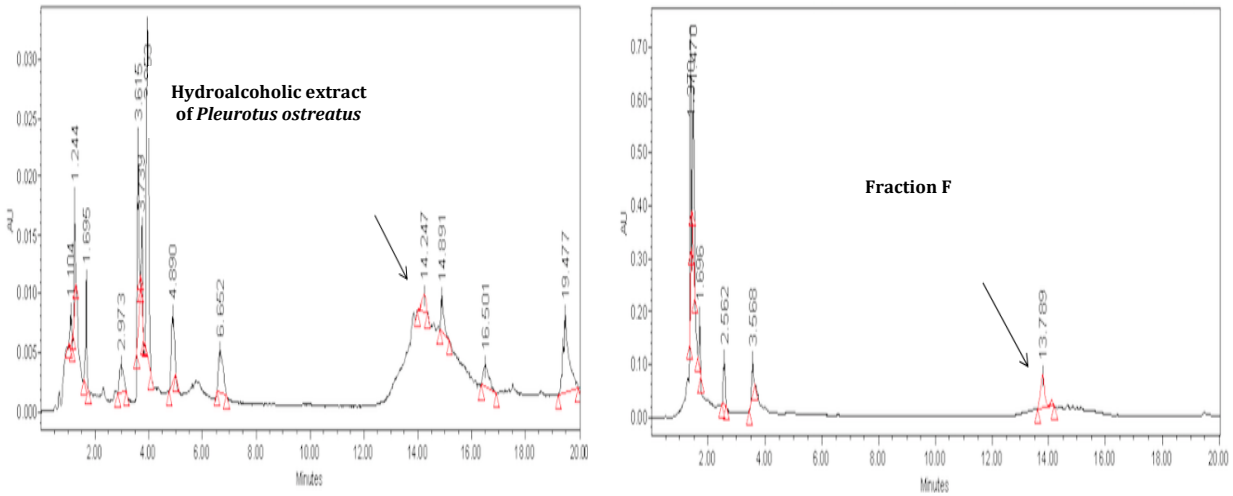
Average (X) and standard deviation (SD) were calculated from three repetitions each. Values followed by a different letter indicate statistical differences (P<0.05) among concentrations and time.

Chemical fractionation of mycelium of *P. ostreatus* yielded 12 fractions and were named A-G as shown in Table 8.

Table 8. Fractionation of hydroalcoholic extract of mycelium of *Pleurotus ostreatus*.

System: CH_2Cl_2-CH_3OH	Fraction	Collected fraction	Yield (g)
100% CH_2Cl_2	1	A	----
90:10	2	B	0.1424
	3		
	4		
80:20	5	C	-----
	6		
70:30	7	D	1.0393
	8		
50:50	9	E	0.2827
	10		
100% CH_3OH	11	F	0.5534
100% CH_3OH	12	G	0.1692

Results of separation of compounds from hydroalcoholic extracts of *P. ostreatus* and fraction "F" are shown in chromatograms of Figures 1-2. The hydroalcoholic extract possessed four probably "active" compounds that were also found in fraction "F". In the latter, the compound responsible for anthelmintic activity can be observed at minute 14 with an absorbance unit of 0.004.



Figures 1 (left) and 2 (right). Chromatograms of hydroalcoholic extract and fraction "F" of mycelium of *P. ostreatus* (ECS-1123) on HPLC at 280 nm with a flow of 0.9 ml/min.

In vitro comparison of two methanolic fractions (FpMeOH and FMeOH) of basidiomes of _P. djamor_ against L_4 larvae of _H. contortus_

Highest mortality rate obtained with the FpMeOH fraction was 84.89 at a concentration of 60 mg/ml at 24 h postexposure, whereas for the FMeOH fraction it was 100% at a concentration of 160 mg/ml at 48 and 72 h post-confrontation (Table 9, 10).

Table 9. *In vitro* evaluation of FpMeOH fraction against *Haemonchus contortus* L_4 larvae at various post-confrontation times**.**

Treatment mg/ml	Mortality (%) ± SD		
	24h	48h	72h
Control (Ivermectina, 5)	100.00 [a] ±0.00	100.00 [a] ±0.00	100.00 [a] ±0.00
60	84.89 [a] ±10.10	52.76 [b] ±35.48	39.19 [b] ±7.70
40	8.25 [b] ±4.30	9.06 [c] ±8.08	38.24 [b] ±6.94
20	0.00 [b] ±6.40	14.91 [c] ±11.96	38.65 [b] ±5.13
10	10.22 [b] ±2.38	6.41 [c] ±1.05	26.71 [bc] ±8.36
5	0.00 [b] ±9.44	10.15 [c] ±7.00	17.03 [cd] ±12.67
2.5	0.00 [b] ±2.29	6.96 [c] ±2.41	10.00 [cd] ±5.83
1.25	0.00 [b] ±2.74	0.00 [c] ±2.43	10.00 [cd] ±9.88
0.625	3.21 [b] ±3.24	5.69 c ±4.03	0.65 [d] ±0.65
0.3125	0.00 [b] ±3.92	1.67 [c] ±4.75	4.41 [d] ±5.59
Hanks	14.39 [b] ±6.37	7.75 [c] ±4.80	4.21 [d] ±3.17
E.C.M.	30.48	109.79	48.86
R2	0.97	0.89	0.95

Same letters in the same column indicate that values do not differ statistically, according to Tukey's test. $P \leq 0.05$. N = 4 (four wells). 10 aliquots (drops) per well.

Table 10. *In vitro* evaluation of FMeOH fraction against *Haemonchus contortus* L_4 larvae at different post-confrontation times.

Treatment mg/ml	Mortality (%) ± SD		
	24 h	48 h	72 h
Control (Ivermectina, 5)	100.00 [a] ±0.00	100.00 [a] ±0.00	99.32 [a] ±1.28
160	96.98 [a] ±2.44	100.00 [a] ±0.00	100.00 [a] ±0.00
80	33.92 [b] ±7.57	27.44 [b] ±13.72	36.15 [b] ±44.07
40	13.33 [c] ±5.32	6.69 [c] ±3.52	0.05 [bc] ±4.04
20	7.41 [c] ±2.43	7.31 [c] ±2.22	32.90 [bc] ±6.64
10	3.85 [c] ±7.10	11.50 [c] ±5.66	28.48 [bc] ±3.82
5	9.94 [c] ±6.80	11.74 [c] ±5.70	24.74 [bc] ±4.64
2.5	6.98 [c] ±6.75	6.89 [c] ±5.54	9.30 [bc] ±17.42
1.25	8.06 [c] ±4.79	11.16 [c] ±6.58	9.55 [bc] ±10.28
0.625	7.77 [c] ±6.97	8.64 [c] ±6.54	0.00 [c] ±1.69
0.3125	6.04 [c] ±4.84	7.89 [c] ±2.76	0.00 [c] ±2.35
Hanks	5.51 [c] ±2.13	3.87 [c] ±3.17	6.24 [bc] ±3.78
E.C.M.	28.27	33.7	205.99
R2	0.97	0.97	0.87

Same letters in the same column indicate that values do not differ statistically, according to Tukey's test. $P \leq 0.05$. N = 4 (four wells). 10 aliquots (drops) per well.

In vitro evaluation of five hydroalcoholic extracts of different depleted substrates of _P. djamor_ against eggs and L_3 larvae of _H. contortus_

Ovicidal activity of EHA-SA06, 10, 11, 12, 13

Results obtained by comparing eggs of *H. contortus* with extracts (EHA-SA), showed a similar tendency in readings carried out at 48 and 72 h. However, biological activity at 72 h was lower in all extracts evaluated except for EHA-SA13 (Figure 3). EHA-SA11 did not show ovicidal activity despite having shared similarity in mass composition with EHA-SA13, which had the strongest and highest activity. On the other hand, spring chip, not present in substrate 11 and if it contained substrate 13, apparently potentiated a biological effect in all EHAs evaluated. Since EHA-SA11, without this agroindustrial waste, did not present significant differences compared to the negative control. EHA-SA13 showed 100% percent inhibition with the 2.5 mg/ml concentration. In fact, of all EHAs evaluated, EHA-SA13 demonstrated strong biological activity, as shown by statistical coefficients (Table 9). On the other hand, EHAs 06, 10 and 12 resulted in variable data with high standard deviations. With low concentrations of EHA-SA06 (0.5 to 2.5 mg/ml), percent inhibition was above 83% at 48 h. However, the reading at 72 h showed that biological activity of EHA-SA06 was not consistent, showing values below half the previous reading. EHA-SA10 was similar to EHA-SA06, showing nematicidal activity at the 48 h reading from concentrations ≥1.25 mg/ml and decreasing its activity in the reading taken at 72 h.

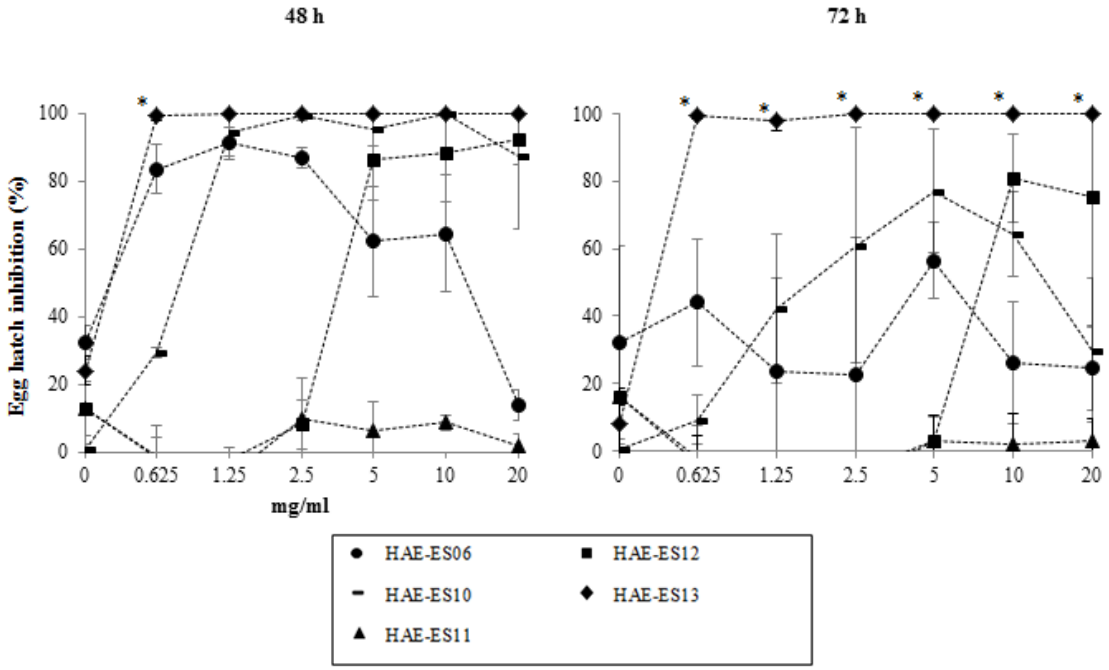


Figure 3. Ovicidal activity of spent mushroom substrates of *Pleurotus djamor* of different composition. Percentages obtained at 48 and 72 h of EHA-SA 06, 10, 11, 12 and 13 are shown. Asterisks show statistically significant differences (Tukey $P<0.05$).

In general, it is observed that data from EHA-SA06 and 10 showed an increase in the standard deviation from the mean at 72 h, perhaps due to natural inhibition of the negative control. EHA-SA12 had ovicidal activity above 10 mg/ml in treatments at 48 and 72 h. However, like EHA-SA06 and EHA-SA10, high coefficients of variation of the averages resulted non-significance of its nematicidal activity by the erratic biological response against *H. contortus* eggs, as well as the high rate of infertility shown in the controls.

It should be mentioned that data used for the analysis of variance in the SAS V9 statistical package were not modified for normalization and thus only one treatment (EHA-SA13) with a high statistical significance (P <0.0001) was obtained at the required levels of analysis.

Nematicidal activity of EHA-SA: 06, 10, 11, 12, 13

Lethal activity obtained in the *in vitro* bioassay is shown in Figure 4. A broad variability of data is shown in the different exposure times with the highest mortality rates for EHA-SA11 obtained at 24 h. At 48 h, fluctuations were observed between EHA-SA13, 12, 06 and 11 that depended on the concentration at which they were evaluated. EHA-SA13 gave the highest biological effectiveness against L_3: 2.5 and 5 mg/ml EHA-SA 12 was the highest. At 10 mg/ml the best was EHA-SA06 and at 20 mg/ml EHA-SA11 showed the highest nematicidal effectiveness. At 72 h of interaction, EHA-SA12 and 13 showed a very similar tendency from the concentration of 0.625 mg/ml. However, between the concentrations of 2.5 to 10 mg/ml, EHA-SA12 presented greater activity in reference to the other extracts. Likewise, similarity of activity between EHA-SA06 and 11 up to the concentration of 10 mg/ml is remarkable. Subsequently EHA-SA11 presented 45% mortality against L_3 larvae without sheath at 20 mg/ml.

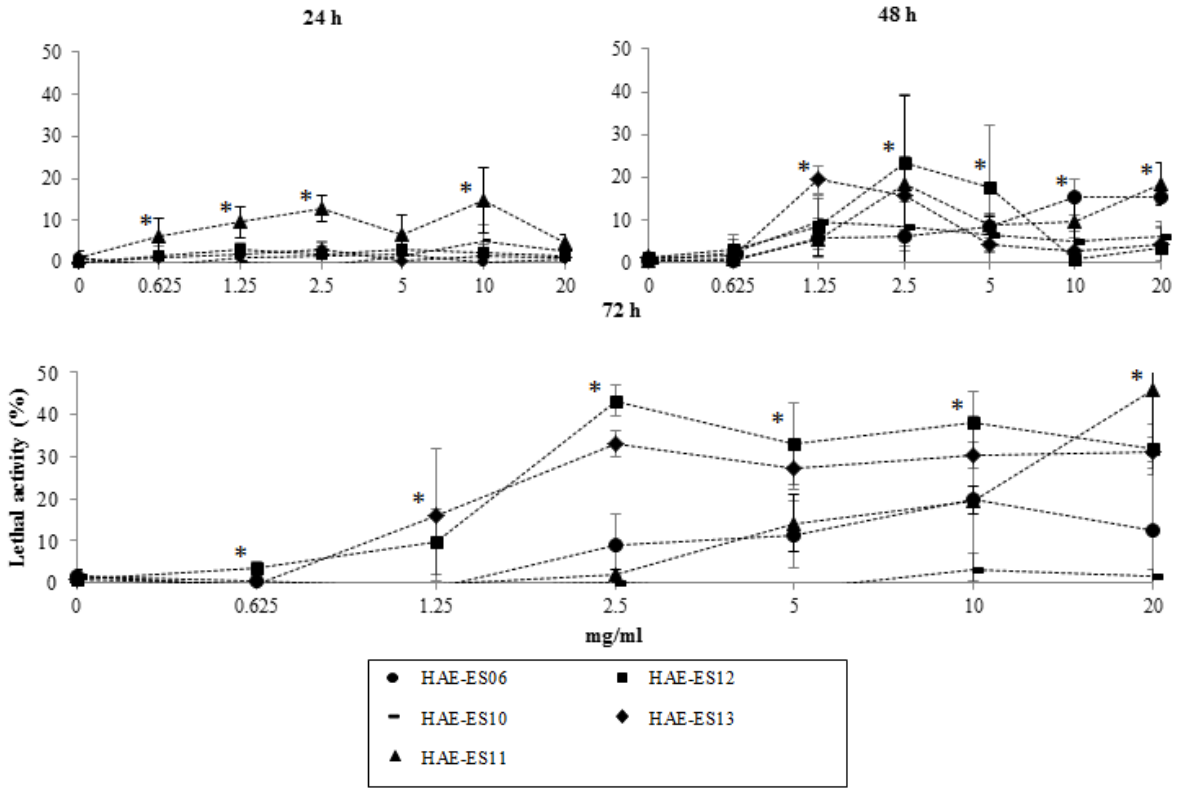


Figure 4. Nematicidal activity of SMS of *Pleurotus djamor*. Percentages obtained at 24, 48 and 72 h of EHA-SA06, 10, 11, 12 and 13 are shown. The asterisks show statistically significant differences (p<0.05).

According to previous results, mortality rates of EHA-SA11 at 24, 48 and 72 h are presented in Table 11. The percentages presented were obtained by adjusting for mortality using the formula of Scneider-Orelli (1972). EHA-SA11 was chosen because it showed higher mortality with respect to 24 h exposure time and confirmed its biological activity at 48 and 72 h. Statistical analysis showed significant difference for the concentration of 20 mg/ml at 72 h exposure.

Table 11. Lethal activity of HAE-SA11 against *Haemonchus contortus* L_3.

HAE-ES11 (mg/ml)	Lethal activity (%)*		
	24 h	48 h	72 h
20	4.47 ^{c,d}	18.20 ^{b}	45.22 ^{b}
10	14.69 ^{b}	9.68 ^{b}	19.28 ^{c}
5	6.53 ^{b,c,d}	8.61 ^{b}	13.91 ^{c,d}
2.5	12.7 ^{b,c}	18.16 ^{b}	1.8 ^{d,e}
1.25	9.51 ^{b,c,d}	5.26 ^{b}	0.00 ^{e}
0.625	6.06 ^{b,c,d}	0.98 ^{b}	0.00 ^{e}
MSE	16.53	65.78	35.89
P value	< 0.0001	< 0.0001	< 0.0001

*Adjusted percentage with the Schneider-Orelli formula (Püntener 1981). Same letters in the same column are not significantly different, Tukey (P< 0.05).

DISCUSSION

In recent decades the study of macromycetes has increased. Extracts from edible mushrooms contain various bio-active components that are important in human and animal health. Miles and Chang (2004) reported that 77% of medicinal mushroom products are derived from commercially grown or commercially harvested fruit bodies, 21% of all products are derived from fungal mycelium and about 2% are derived of filtrates of culture media containing different nutrients. Mycelium and filtered medium are products that are increasingly important, since they meet the requirement of greater safety, quality control and production throughout the year (Valencia and Garín 2012). While considerable information is available regarding human health, little is known about fungal extracts and their interactions in animals and their pathogens. Results obtained from the evaluation of the hydroalcoholic extracts of the mycelium of *P. ostreatus* against different stages of *H. contortus* are encouraging. Additional work is needed to further investigate compound(s) that are responsible for this activity.

The fungus *P. ostreatus* possesses compounds with nematicidal activity, as reported by Kwok *et al*. (1992). They identified a nemato-toxin, "2-trans-acid-decenedioic" that was evaluated against a free-living nematode *Panagrellus redivivus*. Additionally, Stadler *et al*. (1994) identified several molecules corresponding to S-choric acid, linoleic acid and p-anisaldehyde of the fungus *P. pulmonarius* that was responsible for nematicidal activity against a bacteriophage of the nematode *Caenorhabditis elegans.* On the other hand, Satou *et al*. (2008) demonstrated that the nematicidal mechanism of *P. ostreatus* against a nematode of the Diplogastridae family is through linoleic acid peroxide, a molecule that induces reduction of the anterior part (head) of the nematode and causes its death. In addition, Khan *et al*. (2014) stated that the fungus *P. eryngii* possesses the ability to kill *Bursaphelenchus xylophilus*, a nematode that attacks pine wood. Rojas (2013) showed that the same fungus species attacks phytopathogenic nematodes such as *Meloidogyne javanica* and *Heterodera schachtii* through the production of nematode toxins. In the present investigation, a compound was found in fraction "F" that could coincide with that reported by Alam *et al*. (2011). He found that *P. ostreatus* contains several phenolic compounds such as gallic acid, chlorogenic acid, protocatecuic acid and naringenin. Our "F" fraction was obtained with a 100% methanol system with secondary metabolites present being very polar. Our compound was purified by high performance liquid chromatography (HPLC) and it is inferred that this is a phenolic compound. Our work and that reported by Arizmendi *et al.* (2014) shows the fungus *P. ostreatus* possesses bioactive compounds with nematicidal properties both in the fruit body and mycelium. In the future, we plan to continue with the study of *Pleurotus* spp. against various stages of *H. contortus*. In relation to *in vitro* comparison of the two methanolic fractions (FpMeOH and FMeOH) of basidiomes of *P. djamor* against L_4 of *H. contortus*, the main objective of using this stage of larvae was to try to simulate the conditions of the L_4 in sheep abomasum (Schwarz *et al*. 2013, Gadahi *et al.* 2016). Results of the present study demonstrate that FpMeOH and FMeOH fractions possess nematicidal activity. These fractions may have bioactive

compounds that could be used to control the *H. contortus* L_4 stage. It would be necessary to purify and identify compounds by means of different chromatographic and spectroscopic techniques (nuclear magnetic resonance) to elucidate molecules responsible for nematicidal activity of *P. djamor*. Fungi are one of the main generators of biologically active natural products that have a high capacity for use as therapeutic agents for use in mammals (Li and Zhang 2014). Among these compounds more than 200 nematicidal compounds are counted. *In vitro* evaluation of five hydroalcoholic extracts of different *P. djamor* SMS against *H. contortus* eggs and larvae L_3 indicate the composition of the substrates for fungal production are differential in their metabolic response.

Stadler and Sterner in (1998) evaluated nematicidal activity in response to stress inducing stimuli in the fungus, such as physical damage to the basidiome. Similarly, there is information that the substrate composition is important for potentiating the nematicidal activity of EHAs from basidiomes of *P. djamor* (Robledo *et al.* 2015) and that the fungus stage is also important (Valdez 2015). Mycelium has dual activity against *H. contortus* larvae and egg as shown in previous studies using *P. ostreatus* as a productive species (Valdez 2015). This work suggests that the active compounds against *H. contortus* eggs produced by the fungus during its development and that said compounds are stable at extraction temperature and pH, since they remain active when subjected to the chemical maceration procedure. The fungi appear to produce bioactive compounds that are stored in mycelial structures and degradation compounds or extracellular compounds that are excreted into the substrate.

Mushrooms are proteinaceous and the substrate formulations may include cereal straws and various grains that are components of animal diets (Noriega *et al.* 2009, Esqueda-Esquivel and Tosquy-Valle 2007, FIRA, 2015, Rinker 2017). The use of a "nutraceutical" food based on SMS of edible mushroom culture represents a potential method of prevention of nematode infections.

CONCLUSIONS

Hydroalcoholic extracts of the mycelium of *P. ostreatus* and *P. eryngii* showed a minimal nematicidal activity at concentrations used. However, *P. ostreatus* had higher nematicidal activity than *P. eryngii*. The "F" fraction of hydroalcoholic extract of *P. ostreatus* mycelium showed the highest nematicidal activity (25.29%) with respect to other fractions. FpMeOH and FMeOH fractions had the highest L_4 mortality rate of 84.8 and 100, respectively at 24 and 72 h. SMS of *P. djamor* had biological activity against *H. contortus* in its egg stages and L_3. Furthermore, such activity is possibly influenced by the composition and/or the type of substrate. EHA-SA11 was lethal against L_3 with a 45% mortality at 20 mg/ml at 72 h postexposure, but did not affect nematode eggs. In contrast, EHA-SA13 was the most effective against parasite eggs with 100% inhibition starting at 2.5 mg/ml that was determined at CL_{90}= 625 µg/ml.

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PERSPECTIVES

PERSPECTIVES ON MACROMYCETES OF THE TROPICS

Since publication of the first book on tropical fungi by Chang and Quimio (1982) that was devoted to the genera *Volvariella*, *Auricularia* and *Pleurotus*, there have been a few other efforts to disseminate knowledge acquired on this important subject. These include the works of Quimio *et al.* (1990), Quimio (2002) and more recently, Thakur and Thakur (2015), the latter referring to the edible fungi of India. However, given the magnitude of the potential that tropical fungi represent, these efforts are scarce and there is much work to be done.

As mentioned in the introductory chapter of this book, the tropics contain a great diversity of climates that embrace different biological ecosystems. There are 17 megadiverse countries on Earth, of which 15 are precisely located (in their entirety or in a significant part), within the tropical zones (Biodiversity 2014). This includes regions of southern China, north-central Brazil, south-central Mexico, Colombia, most of Africa, India, etc. Although macromycetes are not mentioned specifically, it is very likely that diversity of these organisms in these countries and in general in tropical zones is very high and, likewise, poorly known. Except in China, where great strides have been made to study macromycetes, efforts in other tropical countries for development of mycology in general, and in particular for the study of macromycetes, have been and continue to be very limited. This is reflected in the scarce knowledge and use for humankind of tropical mycobiota.

In this book, we review cultivation techniques for some tropical species such as *Agaricus subrufescens*, *Sparassis latifolia*, *Tremella fuciformis*, *Schizophyllum commune*, *Lepista nuda*, and some promising biotechnological applications of *Auricularia* spp., *Grifola frondosa*, *Pleurotus* spp., and *Volvariella* spp. Likewise, the existence of tropical species of *Agaricus* and *Lentinula* is highlighted. Undoubtedly, this list is only a small sample of the great diversity of macromycetes present in the tropics. It should be noted that there are several species that are not mentioned in this book and that are already commercially grown, such as *Dictyophora indusiata*, *Trametes versicolor*, etc. This is a reflection of the prevailing reality about the scarce knowledge that tropical macromycetes have. A very important effort should be made immediately to train more mycologists in these regions that are able to study and take advantage of these organisms.

Tropical mushrooms are just beginning to be known; however, they are already seriously threatened. In addition to the high rates of deforestation that are prevalent in tropical countries, climate change puts the biological diversity of the planet at high risk, especially the tropical fungi. According to the data of IUCN (2018), of the 11,783 species considered as vulnerable, only 57 belong to the Kingdom Fungi, which shows the lack of studies on this, especially in the tropical zones. Climate change is a serious threat to the stability of terrestrial environments. The increase in temperature threatens to modify the landscape and the environment, a situation that is exacerbated for organisms that depend on the microclimate that is generated by the vegetation of the area, as is the case of fungi. The human influence on climate change is indisputable and potentially irreversible. Today, climate change affects the health of populations around the world. All communities will be affected, although these effects will disproportionately impact those most vulnerable (Watts *et al.* 2017). Climate change will affect not only the tropical zones, but through the increase of temperature, temperate zones will also be affected. This represents both a threat and an opportunity for the fungal diversity of the world. It will be necessary to carry out further studies and work on obtaining and selecting new varieties of fungi capable of resisting higher temperatures as well as those better adapted to use of different substrates.

For this reason, study of tropical macromycetes is urgently needed, because they are vulnerable organisms and because knowledge allows us to take actions and decisions that lead to their best assessment and conservation.

Currently, most commercially grown mushrooms are sold fresh. This is very good from the culinary and nutritional point of view. However, much remains to be explored in terms of new uses and post-harvest management of fungi, for a better exploitation of their qualities. Surely, for nutraceutical and medicinal uses, alternative preparations may be more appropriate and will have to be developed, such as capsules, extracts, powders, etc. If we desire to live in a sustainable society, it is essential to review development of natural products that are friendly to the environment. In that sense, tropical mushrooms and the products that can be obtained from them surely will be very useful to modern production systems and will contribute elements to a society that becomes more aware every day of their impact in the world.

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APPENDIX

APPENDIX 1. LIST OF MUSHROOMS MENTIONED IN THIS BOOK

Agaricus L.

A. abruptibulbus Peck

A. arvensis Schaeff.

A. augustus Fr.

A. benesii (Pilát) Pilát

A. benzodorus Heinem. & Gooss.-Font.

A. bingensis Heinem.

A. bisporus (J.E. Lange) Imbach

A. bisporus var. *burnettii* Kerrigan & Callac

A. bisporus var. *bisporus* (J.E. Lange) Imbach

A. bitorquis (Quél.) Sacc.

A. blazei (Murrill) ss. Heinemann

A. brasiliensis Wasser, M. Diduck, Amazonas & Stamets

A. campestris L.

A. comtulus Fr.

A. dennisii Heinem.

A. deserticola G. Moreno, Esqueda & Lizárraga

A. endoxanthus Berk. & Broome

A. erectosquamosus Linda J. Chen, K.D. Hyde & R.L. Zhao

A. essettei Bon

A. fissuratus F.H. Møller

A. flocculosipes R.L. Zhao, Desjardin, GGuinb. & K.D. Hyde

A. fuscofibrillosus (F.H. Møller) Pilát

A. gemellatus Kerrigan, L.A. Parra, Cappelli & Weholt

A. heterocystis Heinem. & Gooss.-Font.

A. impudicus (Rea) Pilát

A. inapertus Vellinga

A. laeticulus Callac, L.A. Parra, Linda J. Chen & Raspé (≡ *A. laeticolor* Heinem. & Gooss.-Font.)

A. sylvicola (Vittad) Peck.

Agaricus arvensis Schaeff.

Endoptychum depressum Singer & A.H. Sm.

A. leucocarpus Linda J. Chen, Callac, R.L. Zhao & K.D. Hyde

A. magnivelaris Peck *Tricholoma magnivelare* (Peck) Redhead

A. martineziensis Heinem.

A. martinicensis Pegler

A. moelleri Wasser

A. niveogranulatus Linda J. Chen, R.L. Zhao, Callac & K.D. Hyde

A. phaeolepidotus F.H. Møller

A. purpurellus F.H. Møller

A. rufoaurantiacus Heinem

A. semotus Fr.

A. singeri Heinem

A. subtilipes Thongklang, Linda J. Chen, Callac & K.D. Hyde

A. subrufescens Peck

A. subsaharianus L.A. Parra, Hama & De Kesel

A. suthepensis Linda J. Chen, K.D. Hyde & R.L. Zhao

A. sylvaticus Schaeff.

A. sylvicola (Vittad.) Peck

A. trinitatensis R.E.D. Baker & W.T. Dale

A. trisulphuratus Berk.

A. variicystis Linda J. Chen, K.D. Hyde & R.L. Zhao

A. xanthodermus Genev.

A. xantholepis F.H. Møller

A. yucatanensis Ellis & Everth.

A. sect. *Brunneopicti* Heinem.

A. subg. *Minoriopsis* Linda J. Chen, L.A. Parra, Callac, Angelini & Raspé

Agrocybe Fayod

A. cylindraceae (DC.) Maire *Cyclocybe cylindraceae* (DC.) Vizzini & Angelini

Amanita Pers.

Amanita phalloides (Vaill. Ex Fr.) Link

Auricularia Bull.

A. auricula-judae (Bull.) Quél.

≡*Auricularia auricula* (L.) Underw.

A. delicata (Mont. Ex Fr.) Henn.

A. fuscosuccinea (Mont.) Henn.

A. mesenterica (Dicks.) Pers.

A. nigricans Sw. Birkebak, Looney & Sánchez-García (≡*A. polytricha* (Mont.) Sacc.)

Bolbitius Fr.

Cantharellus cibarius Fr.

Clitocybe maxima (P. Gaertn., G. Mey., & Scherb.) P. Kumm. *Clitocybe gibba* (Pers.) P. Kumm.

Conocybe Fayod

Cookeina sulcipes (Berk.) Kuntze

Coprinus Pers.

Cortinarius (Pers.) Gray

Cordyceps Fr.

Cordyceps sinensis (Berk.) Sacc. *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora

Cortinarius speciosissimus Kühner & Romagn. *Cortinarius rubellus* Cooke

Flammulina P. Karst.

Flammulina velutipes (Curtis) Singer

Galerina Earle

Ganoderma P. Karst.

G. applanatum (Pers.) Pat.

G. lucidum (Curtis) P. Karst.

Grifola Gray

G. frondosa (Dicks.) Gray

G. gargal Singer

Hericium Pers.

Hydnopolyporus D.A. Reid

Hypocrea Fr.

Hypsizigus marmoreus (Peck.) H.E. Bigelow

Inocybe (Fr.) Fr.

Inonotus P. Karst.

Lactarius Pers.

Lentinula Earle

Lentinula boryana (Berk. & Mont.) Pegler

Lentinula edodes(Berk.) Pegler

Lentinus Fr.

Lepiota (Pers.) Gray

Lepista nuda (Bull.) Cooke

Morchella esculenta (L.) Pers.

Phallus indusiatus Vent.

Phanerochaete carnosa (Burt) Parmasto

Phellinus Quél.

Pholiota nameko (T. Itô) S. Ito & S. Imai

Pleurotus (Fr.) P. Kumm.

P. abalonus Y.H. Han, K.M. Chen & S. Cheng

P. citrinopileatus Singer

P. cystidiosus O.K. Mill.

P. djamor (Rumph. ex Fr.) Boedijn

P. eryngii (DC.) Quél.

P. nebrodensis (Inzenga) Quél.

P. ostreatus (Jacq.) P. Kumm

P. tuber-regium (Fr.) Singer

Polyporus P. Micheli ex Adans.

Polyporus brumalis (Pers.) Fr.

Poria cocos F.A. Wolf

Psilocybe semilanceata (Fr.) P. Kumm.

Russula Pers.

Schizophyllum Fr.

Sparassis latifolia Y.C. Dai & Zheng Wang

P. cystidiosus O.K. Mill.

Lentinus brumalis (Pers.) Zmitr.

Wolfiporia cocos (F.A. Wolf) Ryvarden & Gilb.

Sparassis crispa (Wulfen) Fr.

Schizophyllum commune Fr.

Trametes Fr. (*Coriolus* Quél.),

T. versicolor (L.) Lloyd

Tremella Pers.

T. fuciformis Berk.

Trichoderma Pers.

T. harzianum Rifai

Volvariella Speg.

V. bombycina (Schaeff.) Singer

V. bombycina var. bombycina (Schaeff.) Singer

V. bombicina var. flaviceps (Murrill) Shaffer

Volvariella volvacea (Bull.) Singer

V. bakeri (Murrill) Shaffer

Wolfiporia cocos (F.A. Wolf) Ryvarden & Gilb.

Appendix 2 – Tropical species of *Agaricus*. Philippe Callac and Jie Chen.
List of 185 species of *Agaricus* newly described since 2000 and their taxonomic placement in the 24 sections of the current system of classification. Sections are ordered like in Table 1. Considered as a doubtful species, *Agaricus dilutibrunneus* R.L. Zhao is excluded from the list.

Agaricus* sect. *Flocculenti Linda J. Chen, K.D. Hyde & R.L. Zhao
Agaricus erectosquamosus Linda J. Chen, K.D. Hyde & R.L. Zhao
Agaricus pallidobrunneus R.L. Zhao
Agaricus* sect. *Brunneopicti Heinem.
Agaricus brunneosquamulosus Linda J. Chen, R.L. Zhao, K.D. Hyde & Callac
Agaricus chiangmaiensis Karunarathna, Guinb. & K.D. Hyde
Agaricus megacystidiatus Karunarathna, Guinb. & K.D. Hyde
Agaricus niveogranulatus Linda J. Chen, R.L. Zhao, Callac & K.D. Hyde
Agaricus pakistanicus H. Bashir, Khalid, L.A. Parra & Callac
Agaricus sordidocarpus Linda J. Chen, Callac & K.D. Hyde
Agaricus sparsisquamosus H. Bashir, S. Hussain, Khalid & H. Ahmed
Agaricus subsaharianus L.A. Parra, Hama & De Kesel
Agaricus toluenolens Callac, Linda J. Chen & K.D. Hyde
Agaricus* sect. *Trisulphurati Heinem.
Agaricus ignicolor R.L. Zhao
Agaricus* sect. *Crassispori R.L. Zhao
Agaricus lamellidistans R.L. Zhao
Agaricus variicystis Linda J. Chen, K.D. Hyde & R.L. Zhao
Agaricus sect. Cymbiformes M.Q. He & R.L. Zhao
Agaricus angusticystidiatus M.Q. He, Desjardin, K.D. Hyde & R.L. Zhao
Agaricus* sect. *Rubricosi R.L. Zhao
Agaricus dolichopus R.L. Zhao
Agaricus kunmingensis R.L. Zhao
Agaricus variabilicolor R.L. Zhao
Agaricus* sect. *Bivelares (Kauffman) L.A. Parra
Agaricus agrinferus Kerrigan & Callac
Agaricus cupressophilus Kerrigan
Agaricus qilianensis S.L. Wei, M.Z. Zhang & R.L. Zhao
Agaricus sinodeliciosus Z.R. Wang & R.L. Zhao
Agaricus sinotetrasporus Y.L. Xi, M.Z. Zhang & R.L. Zhao
Agaricus sipapuensis Kerrigan
Agaricus subsubensis Kerrigan
Agaricus taeniatus Sai F. Li, Shao J. Li & H.A. Wen
Agaricus tlaxcalensis Callac & G.Mata

Agaricus* sect. *Xanthodermatei Singer
Agaricus arizonicus Kerrigan
Agaricus atrodiscus Linda J. Chen, Callac, R.L. Zhao & K.D. Hyde
Agaricus berryessae Kerrigan
Agaricus bisporiticus Nawaz, Callac, Thongklang & Khalid
Agaricus brunneogracilis R.L. Zhao & K.D. Hyde
Agaricus buckmacadooi Kerrigan
Agaricus candussoi L.A. Parra, Angelini & Callac
Agaricus daliensis H.Y. Su & R.L. Zhao
Agaricus deardorffensis Kerrigan
Agaricus exilissimus Linda J. Chen, Callac, R.L. Zhao & K.D. Hyde
Agaricus flavidodiscus L.A. Parra, Angelini & Callac
Agaricus freirei Blanco-Dios
Agaricus fuscopunctatus Thongklang, Linda J. Chen, Callac & K.D. Hyde
Agaricus gregariomyces J.L. Zhou & R.L. Zhao
Agaricus karstomyces R.L. Zhao
Agaricus kriegeri Kerrigan
Agaricus laskibarii L.A. Parra & Arrillaga
Agaricus leptocaulis Kerrigan
Agaricus malangelus Kerrigan
Agaricus melanocarpus R.L. Zhao
Agaricus moelleroides Guinb. & L.A. Parra
Agaricus murinocephalus R.L. Zhao, Desjardin & K.D. Hyde
Agaricus parvitigrinus Guinb. & Callac
Agaricus punjabensis T. Qasim, A. Ashraf & Khalid
Agaricus sinoplacomyces Callac & R.L. Zhao
Agaricus tephrolepidus L.A. Parra, C. Billette, Angelini, G. Mata & Callac
Agaricus tibetensis J.L. Zhou & R.L. Zhao
Agaricus tollocanensis Callac & G. Mata
Agaricus tytthocarpus R.L. Zhao
Agaricus xanthodermulus Callac & Guinb
Agaricus* sect. *Chitonioides Romagn.
Agaricus* sect. *Hondenses R.L. Zhao & L.A. Parra
Agaricus biannulatus Mua, L.A.Parra, Cappelli & Callac

Agaricus grandiomyces J.L. Zhou & R.L. Zhao
Agaricus pusillobulbosus S.Y. Su & R.L. Zhao
Agaricus* sect. *Sanguinolenti Jul. Schäff. & F.H. Møller ex L.A. Parra
Agaricus iranicus Mahdizadeh, Safaie, M. Goltapeh, L.A. Parra & Callac
Agaricus cordillerensis Kerrigan
Agaricus hupohanae Kerrigan
Agaricus thujae Kerrigan
Agaricus* sect. *Bohusia (L.A. Parra) L.A. Parra & R.L. Zhao
Agaricus brunneofibrillosus Kerrigan
Agaricus coniferarum Guinb. & Callac
Agaricus crassisquamosus R.L. Zhao
Agaricus lusitanicus Callac, L.A. Parra & A. Tancrède
Agaricus* sect. *Nigrobrunnescens K.R. Peterson, Desjardin & Hemmes
Agaricus erythrosarx T. Lebel
Agaricus caballeroi L.A.Parra, G.Muñoz & Callac
Agaricus collegarum L.A. Parra, Wisman, Guinb., Weholt, Musumeci & Geml
Agaricus desjardinii Z.R. Wang, K.D. Hyde & R.L. Zhao
Agaricus laparrae Kerrigan
Agaricus masoalensis L.A. Parra, Wilhem & Callac
Agaricus padanus Lancon.
Agaricus* sect. *Agaricus L.
Agaricus erindalensis Kerrigan
Agaricus flavicentrus Karunarathna & K.D. Hyde
Agaricus gastronevadensis Kerrigan
Agaricus griseocephalus Kerrigan
Agaricus incultorum Kerrigan
Agaricus inilleasper T. Lebel
Agaricus* sect. *Amoeni Callac & R.L. Zhao
Agaricus amoenomyces R.L. Zhao
Agaricus gratolens Pradeep & R.L. Zhao
Agaricus suthepensis Linda J. Chen, K.D. Hyde & R.L. Zhao
Agaricus* sect. *Rarolentes Kerrigan
Agaricus albosquamosus Linda J. Chen, K.D. Hyde & R.L. Zhao
Agaricus butyreburneus Kerrigan, Guinb. & Callac
Agaricus hanthanaensis Karunarathna & K.D. Hyde
Agaricus leucolepidotus Linda J. Chen & R.L. Zhao
Agaricus* sect. *Spissicaules (Heinem.) Kerrigan
Agaricus bellanniae Guinb., Kerrigan & M. Kuo
Agaricus lanipedisimilis Callac & R.L. Zhao
Agaricus litoraloides R.L. Zhao
Agaricus planipileus R.L. Zhao
Agaricus* sect. *Subrutilescentes Kerrigan
Agaricus brunneopileatus Callac & R.L. Zhao
Agaricus catenariocystidiosus R.C. Dai & R.L. Zhao
Agaricus inthanonensis Linda J. Chen, K.D. Hyde & R.L. Zhao
Agaricus linzhiensis R.L. Zhao
Agaricus parasubrutilescens Callac & R.L. Zhao
Agaricus thiersii Kerrigan & Vellinga
Agaricus vinosobrunneofumidus Kerrigan
Agaricus* sect. *Arvenses (Konrad & Maub.) Konrad & Maub.
Agaricus cruciquercorum Kerrigan
Agaricus didymus Kerrigan
Agaricus diospyros B. Ortiz, Kerrigan & Skulan
Agaricus eburneocanus T. Lebel
Agaricus flocculosipes R.L. Zhao, Desjardin, Guinb. & K.D. Hyde
Agaricus gemellatus Kerrigan, L.A. Parra, Cappelli & Weholt
Agaricus guizhouensis Y. Gui, Zuo Y. Liu & K.D. Hyde
Agaricus indistinctus L.A. Parra & Kerrigan
Agaricus julius Kerrigan
Agaricus longistipes Y. Gui, Zuo Y. Liu, Callac, L.A. Parra & K.D. Hyde
Agaricus megalocarpus Y. Gui, Zuo Y. Liu, Callac, L.A. Parra& K.D. Hyde
Agaricus mesocarpus Kerrigan
Agaricus moronii Kerrigan
Agaricus nanaugustus Kerrigan
Agaricus ornatipes A. Mua, M. Casula & M. Sanna
Agaricus reducibulbus Kerrigan
Agaricus sandianus Kerrigan
Agaricus subantarcticus Geml, Laursen & D.Lee Taylor
Agaricus subtilipes Thongklang, Linda J. Chen, Callac & K.D. Hyde
Agaricus* sect. *Minoriopsis Linda J. Chen, L.A. Parra, Callac, Angelini & Raspé
Agaricus argenteopurpureus L.A. Parra, Angelini & Callac
Agaricus* sect. *Kerrigania L.A. Parra, Angelini, B. Ortiz, Linda J. Chen & Callac
Agaricus globocystidiatus Drewinski & M.A. Neves
Agaricus porphyropos L.A. Parra, Angelini & B. Ortiz
Agaricus* sect. *Leucocarpi Linda J. Chen & Callac
Agaricus leucocarpus Linda J. Chen, Callac, R.L. Zhao & K.D. Hyde
Agaricus* sect. *Pantropicales L.A. Parra, Angelini, B. Ortiz, Linda J. Chen & Callac
Agaricus lodgeae L.A. Parra, Angelini & B. Ortiz

Agaricus* sect. *Minores (Fr.) Henn.
Agaricus armandomyces M.Q. He & R.L. Zhao
Agaricus arrillagarum L.A. Parra, S. Serrano & Geml
Agaricus badioniveus Linda J. Chen, R.L. Zhao & K.D. Hyde
Agaricus blatteus M.Q. He & R.L. Zhao
Agaricus bonisquamulosus M.Q. He & R.L. Zhao
Agaricus brunneolutosus Linda J. Chen, Karunarathna & K.D. Hyde
Agaricus callacii L.A. Parra, Iglesias, Fern. Vincente & Oyarzabal
Agaricus campbellensis Geml, Laursen & D.Lee Taylor
Agaricus catenatus M.Q. He & R.L. Zhao
Agaricus cerinipileus M.Q. He & R.L. Zhao
Agaricus chartaceus T. Lebel
Agaricus coccyginus M.Q. He & R.L. Zhao
Agaricus colpetei T. Lebel
Agaricus dilatostipes M.Q. He & R.L. Zhao
Agaricus edmondoi L.A. Parra, Cappelli & Callac
Agaricus elongatestipes M.Q. He & R.L. Zhao
Agaricus fimbrimarginatus Linda J. Chen, Callac & K.D. Hyde
Agaricus flammicolor Linda J. Chen, Callac, R.L. Zhao & K.D. Hyde
Agaricus flavopileatus Linda J. Chen, Karunarathna & Callac
Agaricus friesianus L.A. Parra, Olariaga & Callac
Agaricus fulvoaurantiacus Linda J. Chen & Karunarathna
Agaricus gemlii L.A. Parra, Arrillaga, M.Á. Ribes & Callac
Agaricus gemloides M.Q. He & R.L. Zhao
Agaricus globosporus M.Q. He & R.L. Zhao
Agaricus greuteri L.A. Parra, Cappelli & Kerrigan
Agaricus jacobi L.A. Parra, A. Caball. & Callac
Agaricus jingningensis M.Q. He & R.L. Zhao
Agaricus kerriganii L.A. Parra, B. Rodr., A. Caball., M. Martín-Calvo & Callac
Agaricus lamelliperditus T. Lebel & M.D. Barrett
Agaricus luteofibrillosus M.Q. He, Linda J. Chen & R.L. Zhao
Agaricus luteopallidus Linda J. Chen, Karunarathna, R.L. Zhao & K.D. Hyde
Agaricus mangaoensis M.Q. He & R.L. Zhao.
Agaricus marisae L.A. Parra & Callac
Agaricus matrum L.A. Parra, A. Caball., S. Serrano, E. Fern. & Callac
Agaricus megalosporus Linda J. Chen, R.L. Zhao, Karun. & K.D. Hyde
Agaricus microviolaceus M.Q. He & R.L. Zhao
Agaricus minipurpureus M.Q. He & R.L. Zhao
Agaricus neimengguensis M.Q. He & R.L. Zhao
Agaricus parvibicolor Linda J. Chen, R.L. Zhao & K.D. Hyde
Agaricus parvibrunneus M.Q. He, K.D. Hyde & R.L. Zhao
Agaricus patris Linda J. Chen, Callac, K.D. Hyde & R.L. Zhao
Agaricus pietatis L.A. Parra & A. Caball.
Agaricus pseudominipurpureus M.Q. He, K.D. Hyde & R.L. Zhao
Agaricus pseudopallens M.Q. He & R.L. Zhao
Agaricus pseudopurpurellus M.Q. He & R.L. Zhao
Agaricus purpureofibrillosus Linda J. Chen, R.L. Zhao & K.D. Hyde
Agaricus purpureosquameus M.Q. He & R.L. Zhao
Agaricus robustulus Linda J. Chen, Callac, L.A. Parra, K.D. Hyde & De Kesel
Agaricus rufifibrillosus M.Q. He & R.L. Zhao
Agaricus rufipileus M.Q. He & R.L. Zhao
Agaricus sodalis Linda J. Chen, R.L. Zhao & K.D. Hyde
Agaricus stevensii Kerrigan
Agaricus yanzhiensis M.Q. He, K.D. Hyde & R.L. Zhao
Unclassified species (*insertae sedis* and species lacking sequence data)
Agaricus evertens Kerrigan
Agaricus haematinus K.D. Hyde & R.L. Zhao
Agaricus heinemannii Albertó & G. Moreno
Agaricus nigrogracilis R.L. Zhao
Agaricus pachydermus T. Lebel
Agaricus patialensis M. Kaur & Harw. Kaur
Agaricus pseudolangei K.D. Hyde & R.L. Zhao
Agaricus stijvei de Meijer
Agaricus tennesseensis Kerrigan

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