3 INTRODUCTION

3.1 GENERAL ASPECTS OF EDIBLE MUSHROOMS

Fungi are defined as non-photosynthetic hyphal eukaryotes and related forms [1]. All edible fungi are SAPROPHYTES. Today about 7000 species possess varying degrees of edibility, and more than 3000 species may be considered prime edible species, of which only 200 species have been experimentally grown, 100 economically cultivated, approximately 60 commercially cultivated, and about 10 species cultivated on an industrial scale [2].

The use of lignocellulosic materials, which provide a sustainable biomass resource for the growth of edible and medicinal mushrooms, is of great environmental importance for it recycles organic waste, thereby playing a role in controlling problems of pollution. Edible fungi are primary agents of decomposition, important in cycling a variety of elements such as carbon, nitrogen, and oxygen.

Edible mushrooms have been used as a food alternative for more than 2000 years in Europe and Asia, where they were cultivated for consumption under primitive conditions [3]. The efficiency of fungi in converting SUBSTRATE to protein is far superior to that of several plants and even animals. Mushrooms are low in calories, sodium, fat and cholesterol, while rich in protein, carbohydrate, fibre, vitamins and minerals. Furthermore, some species also produce medicinally effective products (nutriceuticals). Consumption of mushrooms has a positive effect on the general human health. These nutritional properties make mushrooms a very good dietary food [4].

3.1.1 BIOLOGY

Edible mushrooms are filamentous, composed of septate hyphae. The hyphae (fungal cells) are divided by cross-walls into compartments which typically contain several nuclei. Their cell walls commonly contain the polysaccharide chitin along with other polysaccharides, lipids, and proteins. Most commercially grown mushrooms are basidiomycetes, characterised by the presence of clamp connections apparently linking adjacent cells of hyphae and basidiospores actively launched from the basidium [1].

In contrast to animals most mushroom species are haploid. The diploid phase is normally transient and restricted to the basidium where meiosis occurs. The role of the edible fruiting bodies is the production of large numbers of spores by means of which dispersal occurs. The spores are borne on the gills below the cap, and a stalk raises the fruit body above the ground to facilitate spore dispersal by air currents.

3.1.2 PHYSIOLOGY

Hyphae can grow over, into, or through the substrate by their extension. This extension occurs at the tips of the hyphae. Nutrients are absorbed from the substratum, while growth, nuclear division and hyphal branching occur to give an approximately circular colony that increases in diameter at a uniform rate [1]. The hyphae of a growing colony are termed a MYCELIUM (Fig. 1).

Fig. 1. Mycelium growth in *Lentinula edodes*

With the help of extracellular enzymes, like cellulases and laccases, fungi degrade many types of macromolecules, such as cellulose, hemicellulose, lignin, protein, and others, present in the substrate. Their main nutritional and physical requirements are carbon, nitrogen, minerals (S, P, K, Mg, Fe, Zn, Mn, Cu, Mb, Ca), vitamins (thiamine, biotin, B_3, B_5), temperature, light, moisture, aeration, and gravity [2].

3.2 Mushroom biotechnology in Mexico

Biotechnology, applied genetic engineering, can be defined as the pursuit of manipulative genetics and molecular biology in hopefully practical directions [1]. Mushroom cultivation presents an economically important biotechnological industry that has markedly expanded all over the world in the past few decades. Mushroom growing has many advantages: no arable land is needed, agricultural waste is converted into fertilizers and soil conditioners, it is income-generating and the mushrooms provide an extra source of protein and valuable vitamins and minerals [5].

Commercial production of edible mushrooms in Mexico has a great importance from a social, economical and ecological point of view. It generates an annual estimate of 150 million dollars and around 20 000 jobs, both direct and indirect. The volume of produced mushrooms is close to 38 700 tonnes a year, obtained from 386 000 tonnes of diverse agroindustrial by-products, accelerating their biodegradation and recycling in nature [6]. Due to their high nutritional value, approximately 360 tonnes of protein are generated [7].

Although several species in Mexico could potentially be cultivated commercially, their cultivation has only been done in an experimental fashion, mainly for research in biotechnology institutes. The joint efforts of the scientific and the commercial factions in

Mexico would promote the constant improvement of production systems [8] and create an industry with enormous exportation potential. Present commercial production has been estimated in 48,985 tonnes of fresh mushrooms (*Agaricus, Pleurotus, Lentinula*) per year, involving about 30,000 jobs and having an economic significance in excess of 120 million dollars [9].

Latin American mushroom growers have succeeded in adapting European, North American and/or Asian technologies to the local conditions of each country, promoting consumption and marketing of fresh and processed mushrooms, and training mushroom farm workers. Within Latin America, Mexico comprises one of the leading countries (58.96%) when it comes to mushroom production. Total exports in the year 2000 reached 1,602 tonnes [9].

The research work in several biotechnological institutions (includng the College of Postgraduates) is focused on strain fertility, genetic characterization, mushroom breeding and the promotion of edible mushroom rural production which brings several benefits. Another important contribution of the scientific party are culture collections, which represent a vast potential source of biosynthetic products and genes available for genetic engineering [9].

Main efforts are concentrated on developing strains which show high yields, tolerance to high fruiting temperatures, and resistance to competitor moulds. Further genetic improvement considers the post-harvest quality of fruit bodies, increasing degradation efficiency of mushrooms on growing substrates, and isolation of metabolic products with potential importance for the industry [10]. Mushroom biotechnology will certainly have a further contribution to Mexico’s sustainable development.

3.3 Case study of *Lentinula edodes*

Also known as black forest mushroom and shiitake, *L. edodes* is the second most widely cultivated mushroom in the world. Over the last few decades *L. edodes* has emerged second only to *Agaricus bisporus* as the most popular edible mushroom owing to its flavor, as well as its nutritional (APPENDIX 11.2) and medicinal value. The cultivation of shiitake (Shii – tree species *Castanopsis cuspidata* in which it grows on nature; 'take' – japanese word for mushroom) [11] constitutes the world's largest wood bioconversion process.

L. edodes occurs naturally throughout Asia. A similar species, *L. boryana*, occurs in sub-tropical America. Shiitake's natural habitat is dead hardwood logs in a warm, moist climate. It can be traced to the Cretaceous period, over one hundred million years ago [12]. As a member of the Agaricales order (Fig. 2), *L. edodes* is a wood-decaying mushroom that presents gills, and an attractive texture and flavor.

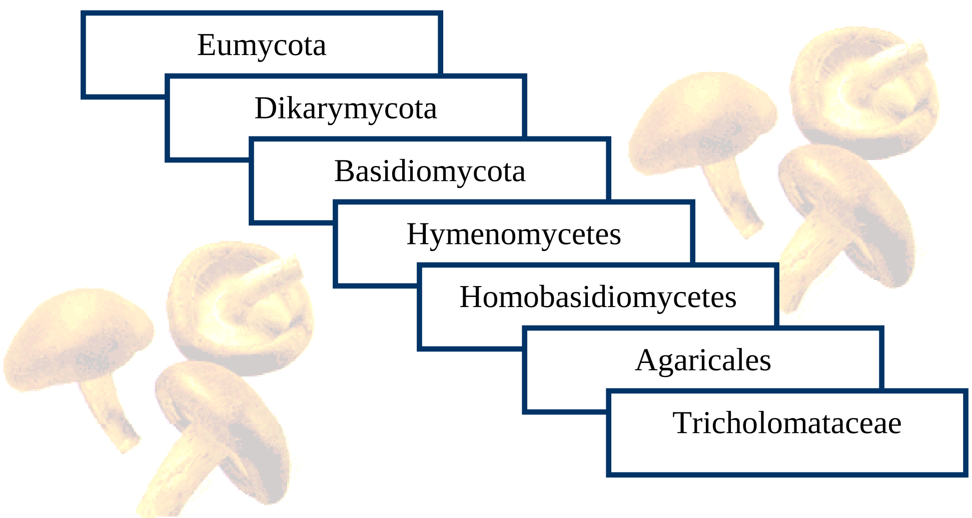

Fig. 2. *Lentinula edodes* classification scheme[1]

The shiitake mushroom is grown mainly in Japan, with an annual production of about 200 000 tonnes a year. Today, a steadily increasing market for fresh shiitake outside of Asia has created a demand for localized shiitake production in many new areas. In 1997, the production worldwide was more than 1,564,000 MT with almost 88.8% produced in China [2]. The production of this mushroom has now spread to the US, Australia, Canada, Brazil, and a few European countries. It is expected that more countries will grow this mushroom, and it could surpass *A. bisporus* and rank in first place for cultivated edible mushrooms worldwide in the near future.

Shiitake is a model among functional mushrooms for extensive research of its bioactivity leading to the isolation of pure compounds (Table 1) which have pharmaceutical effects [13]. Some of these effects have been attributed to a specific polysaccharide (Lentan) or polypeptide (Lenthionine). There may be synergistic effects between these compounds, and between them and other "inactive" compounds in the mushroom.

Compound	**Effect (s)**	**Type of compound**	**Activity**
Eritadenine	Lowers cholesterol Antiviral	Adenine derivative	Accelerates cholesterol metabolism and excretion
Ac2P	Antiviral	Polysaccharide	Inhibits viral replication
Virus-like particles	Antiviral Antitumor	Double stranded RNA	Induces interferon production
KS-2	Antitumor Antiviral	Polysaccharide	Induces interferon production
Lentinan	Antitumor	Polysaccharide	Stimulates T-helper cells in immune system
LAP1	Antitumor	Polysaccharide	Immune systeme modulator
Polyphenol oxidase	Antitumor	Protein	Unknown
Unknown	Reduces blood coagulation	Possibly nucleo -sides or -tides	Inhibits platelet aggregation
Cortinellin	Antibacterial	Unknown	Broad spcetrum antibiotic
Unknown	Antifungal	Disulfide	Unknown
FBP (Fruiting body protein)	Antiviral	Protein	Inhibits viral infection in plant

Table1. Biologically active compounds found in shiitake [10]

3.3.1 LIFE CYCLE

The life cycle of shiitake (Fig. 3) begins when a mature mushroom sheds basidiospores into the air and they are dispersed by the wind. Basidiospores (n) are thin-walled and perish rapidly when exposed to sunlight. Most of them die; however, those that land on a suitable substrate may, under proper conditions, germinate and establish a new colony. When the basidiospore germinates, it produces a new hyphal tip which grows into primary mycelium. If the emerging mycelium does not encounter a suitable food suply, it uses up the nutrient reserves in the spore and then dies. However, if the primary mycelium finds a suitable substrate, it could increase in size indefinitely. Primary mycelium of shiitake can not produce any fruiting bodies. To develop secondary mycelium, two primary hyphae (monokaryons), containing compatible nuclei, must grow together to form a dikaryon (n + n). The secondary mycelium resulting from this mating can produce mushrooms and complete the life cycle [10].

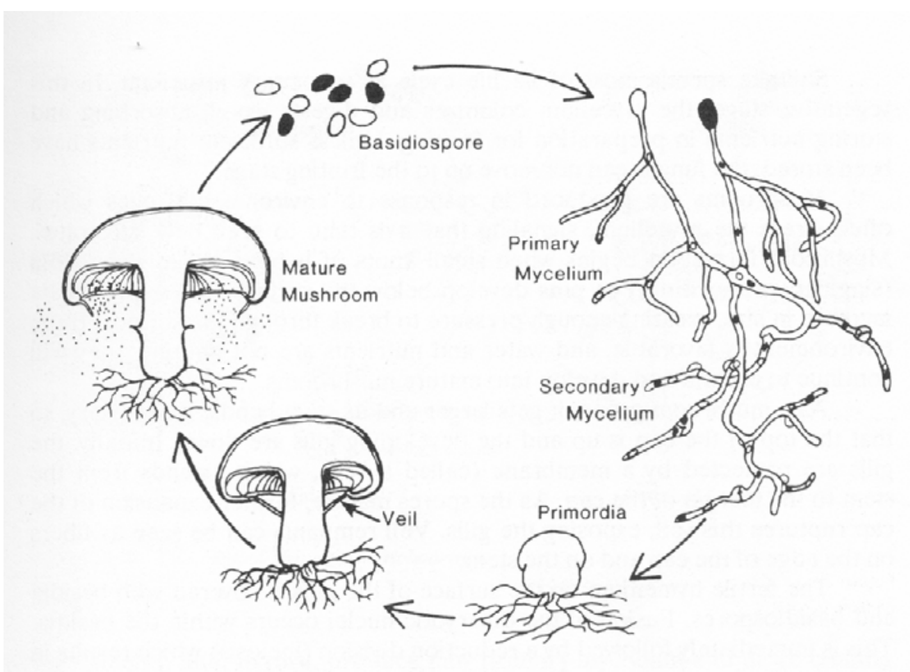

Fig. 3. Shiitake life cycle [10]

The sexual process in *Lentinula*'s life cycle involves three key steps: cell fusion (PLASMOGAMY), nuclear fusion (KARYOGAMY) in the basidium (2n), and meiosis. *Lentinula* is HETEROTHALLIC, with a mating system that prevents reproduction between genetically identical cells. Mating occurs between morphologically identical monokaryons by hyphal fusions and (reciprocal) exchange of nuclei [14]. Shiitake has four mating types which are compatible only in certain combinations. The ability of two monokaryons to grow together and exchange nuclei is controlled by multiallelic genes at two unlinked loci (A and B) accounting for their tetrapolar or bifactorial mating-type system [15]. The consequence of these primary controls of fertility is the promotion of outbreeding.

3.3.2 CULTIVATION PARAMETERS

Shiitake grows on a number of broad-leaved trees (hardwoods), mostly in the oak family (Fagaceae). These include oak (*Quercus*), beech (*Fagus*), chestnut (*Castenea*), hornbeam (*Carpinus*), and chinkapin (*Castanopsis*) in the Fagaceae and other families [10]. Cultivation has been practised for a very long time, but the methods used have become more sophisticated over the past century. By adapting traditional methods and developing new ones, growers are successfully producing shiitake at many different scales.

Cultivation should always focus on creating a selective nutrient base for the mushroom and managing the environment to favor mushroom growth and development. Key environmental factors which influence the growth of shiitake are temperature (growth at 24°-20°C and pinning at 10°-16°C), humidity, evaporation, moisture content in the substrate, light (370-420nm; 180-940 lux), pH (3.5-4.5), and gas concentration (growth at 0.6% CO_2 and fruiting at 0.2% CO_2) [10]. Shiitake can be commercially cultivated on one of two types of substrate: logs or a particulate medium consisting mostly of sawdust.

Shiitake has traditionally been cultivated on hardwood logs (Fig. 4). Growers use SPAWN to introduce the shiitake fungus into the logs on which the crop will be produced. The logs are inoculated with this spawn and achieve mycelial development in one or more years, depending on the wood and the inoculum used. The logs are then transferred to an environment suitable for fruiting (cool, humid, well-sheltered glade). Composed of sawdust and supplemented with millet and wheat bran, synthetic logs may produce three to four times as many mushrooms as natural logs – in one-tenth of the time [16].

In many ways, shiitake cultivation on sawdust is like cultivation on logs. The response of shiitake to the environment is identical. The fungus goes through the same stages of growth: INOCULATION, incubation, induction, pinning, fruiting and resting. It is different, however, due to its particulate nature. This results in higher yields (BIOLOGICAL EFFICIENCY) in a shorter period of time (6 to 9 months).

Substrate preparation (Fig. 4) includes sawdust (from hardwoods) and nutritional supplements (nitrogen and carbohydrates). Substrate must be sterilized prior to inoculation to kill all other microorganisms, creating a selective substrate [10]. Selectivity of the substrate depends on: available nutrients, compactness, water content and water activity, pH, microbial activity, quality of mixture, previous fermentation and/or heat treatment [5]. The optimum temperature for fruiting is generally lower than the optimum temperature for mycelial growth. Removal of excess CO_2 and other self-inhibitory volatile metabolites by

Fig.4. Cultivation on hardwood logs (left) and particulate substrate (middle and left image)

ventilation is also very important for morphogenesis in mushrooms. Nutrients may also serve as stimulus to fruiting [17].

The major trend has been the growth of *Lentinula* in plastic bags (Fig. 5) containing a variety of substrates. The plastic bag cultures can be grown in mushroom houses in which environmental conditions can be controlled. The use of plastic bag culture technique and utilization of waste lignocellulosic materials for substrate (sustainable) rather than wood logs are significant factors in extending *Lentinula* production to countries where it has not previously been produced [2]. With good mushroom strains, suitable compost material, and proper processing of the compost, as well as optimum growing conditions, a high and reliable yield of mushrooms can be obtained.

Fig.5. Cultivation on lignocellulosic materials in polypropylene plastic bags

3.4 Genetic diversity and potential for Genetic Improvement

Much effort has been directed at strain improvement in industrial microorganisms, but not in edible fungi. The genetics and breeding potential of *Lentinula* have not been as extensively investigated as the commercial importance of this mushroom merits. Cultivated mushrooms require simultaneous improvement of a range of characteristics expressed in a biologically complex structure, complicating the genetic enhancement process. Better information on the degree and distribution of genetic variation is essential for developing more efficient ways of evaluating and conserving biodiversity [18], and developing genetically improved strains.

Survival of species in nature under changing environmental conditions requires genetic variation. Commercial cultivars have been used for long periods of time and have limited genetic variability. Future breeding for improved cultivars needs the genetic variation of wild collected strains [2]. The extent to which a fungal species varies in nature can be determined only by studying many strains from different localities. To obtain a true picture of genomic variation within a species, it is important to make genomic analysis of commercial cultivars as well as strains collected from diverse localities in nature.

Strains isolated from nature may differ in morphology, physiology (different growth rates and differing amounts of metabolic products), chromosome number, nucleic acids and proteins, etc. Differences in molecular detail between strains are proving valuable in elucidating the genetic behaviour of natural populations. With selection, optimum combinations of genes controlling characteristics of commerical importance are brought together. Genetic improvement seeks tendencies for such characteristics as stability, high yield, and quality.

Fungi possess features that render many ideal for genetic research. *L. edodes*, for instance, can be grown in pure culture under controlled conditions (minimized environmental input), has genes that are expressed in the haploid phase making mutant alleles of such genes readily detectable and requires simple nutrients. By cross-breeding, favorable characteristics of different species can be combined. Since *Lentinula* is an heterothallic species it is easier to breed than homothallic species, since it is relatively easy to separate spores from different strains and let them fuse. The strains can be compared in factors like growth rate, density, fruiting ability, fruiting temperature range, yield, etc. Other features to select for are size, colour, form, keeping ability, sensitivity to CO_2, spore production, resistance to bruising, concentrations of medicinal compounds, etc. The objective of breeding is to combine favourable characteristics of different strains. Such a combination is often referred to as a HYBRID strain [5].

If improvement is sought by HYBRIDISATION of strains with contrasting characteristics, the most straightforward means is to use a sexual reaction between compatible strains. The procedure is to mate the strain with the desired characteristic with a compatible strain of a cultivar with good commercial qualities. Selection and backcrosses with this cultivar will eventually place the characteristic into the genome of the cultivar, provided that the characteristic in question is simply inherited. What will have occurred is recombination through meiosis. By means of recombination, favorable characteristics found either in wild strains or obtained following mutagenic treatment can be brought together in one organism.

Comparative studies of the nucleotide sequences of ribosomal DNA (rDNA) genes provide a means for analyzing phylogenetic relationships over a wide range of taxonomic levels. The internal transcribed spacer region or ITS region (APPENDIX 11.3) of the nuclear rDNA repeat units evolve fastest and may vary among species withing a genus or among populations [19]. Genetic variability for mushroom yield and quality is necessary when it comes to the genetic improvement of shiitake. Mushroom strains may differ for traits that can be recombined through crosses in conventional genetic breeding, or they can be submitted to protoplast fusion techniques or to transformation with cloned genes using recombinant DNA technology. Thus the importance of assessing the genetic variability of the strains deposited in a germplasm bank.