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Mahendra Rai George Kövics
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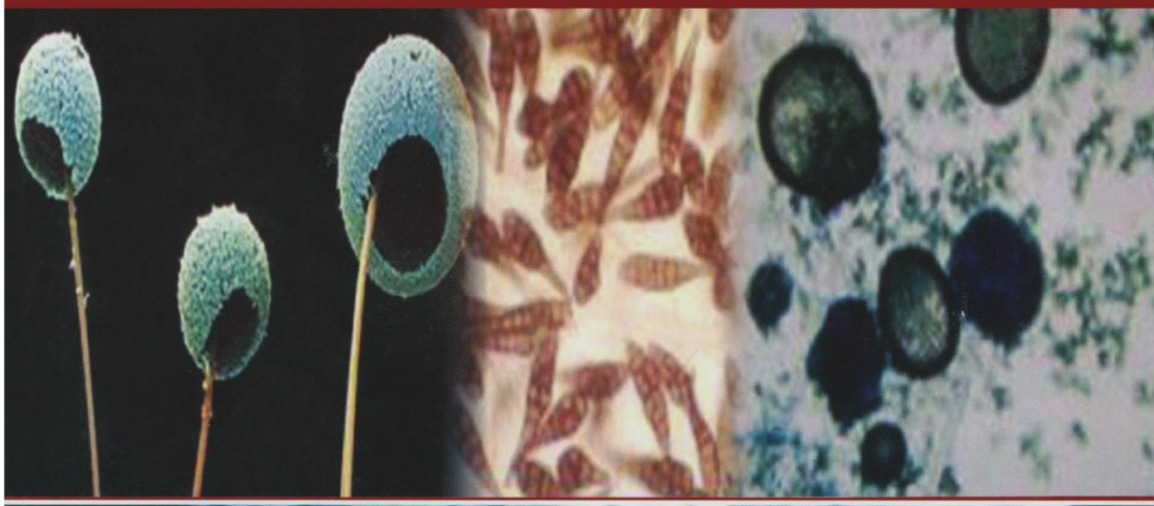

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Preface

The people have been using fungi since ancient times. On one hand fungi are responsible for causing plant and human diseases, while on the other hand they are beneficial to human kind. There have been tremendous biotechnological advancement in the field of fungi in the last two decades. Various applications of the fungi include drugs, dyes, single-cell protein and growth promoters. Advancement in the field of molecular biology, proteomics and genomics have unravelled various doubts and provided new insights in the field of genetic improvement, transformations and phyllogenetic relationship of different genera and species. Fungi are not only involved in production of single-cell protein, wine and beer production and antioxidants but also used for bioremediation, growth promotion, as biosensors and fabrication of eco-friendly silver and gold nanoparticles. Some of these issues have been addressed in the present book.

The present book is aimed to provide the readers with current trends in the field of Mycology in general and fungal biotechnology in particular.

The book would be of utmost importance to students, researchers and teachers of botany, mycology, microbiology, medical microbiology, fungal biotechnology and nanotechnology. The readers should find the book full of information and reader friendly.

We are thankful to all the contributors for submission of their valuable manuscripts. MKR wishes to thank his students- Ravindra Ade, Avinash Ingle, Dnyaneshwar Rathod, Alka Yadav, Vaibhav Tiwari, Jayendra Kesharwani and Swapnil Gaikwad for help in editing and typesetting.

Mahendra Rai
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CHAPTER 1

BETTER YEAST FOR BETTER WINE - GENETIC IMPROVEMENT OF *SACCHAROMYCES CEREVISIAE* WINE STRAINS

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Introduction

The yeast species *Saccharomyces cerevisiae*, commonly called 'wine yeast', 'bakers yeast', 'brewers yeast' or 'distillers yeast' is the main yeast responsible for alcoholic fermentation and has been used for centuries in wine making, baking, brewing and distilling. With the emergence of molecular genetics and genomics, the industrial importance of *S. cerevisiae* continuously extended, providing a tremendous future potential for the development of genetically modified yeast strains (GMY) for the biofuel, bakery and beverage industries or for the production of enzymes and pharmaceutical products.

At present, most of the European wine production relies on the use of selected pure yeast cultures as an oenological practice to produce wine with desirable organoleptical characteristics and to guarantee the homogeneity of successive vintages. These yeast strains were selected from the fermentative flora of a given viticultural region mainly due to their good fermentation performance. There is considerable genetic variation within this species, since different strains of *S. cerevisiae* can vary significantly in their fermentative behavior and the production of compounds that benefit the sensory quality of wine. The accumulated knowledge of the *S. cerevisiae* cellular biology, physiology, biochemistry and genetics, in combination with intensive genomics and proteomics research, will illuminate phenotypic variation in natural populations.

Classical strain improvement approaches have a long-standing history of successful application and rely on repeated cycles of genetic diversity creation

through mutagenesis and/or genetic recombination followed by selection or screening of the desired phenotypes. Targeted genetic manipulation were undertaken even long before the publication of the *S. cerevisiae* genome sequence (Goffeau *et al.*, 1996). More recently, classical methods of strain selection became blended with the latest whole cell engineering approaches such as genome shuffling or evolutionary engineering, that mimic the principles of natural whole genome evolution in a laboratory setting. These procedures provide a promising means for the design of multiple complex, polygenic phenotypes in industrial yeasts, when coupled to high throughput screening and analytical technologies such as robotic miniaturization of assays. In parallel, unlocking the transcriptome, proteome and metabolome complexities in the post-"omics" era, decisively contributes to the knowledge about the genetic make-up of commercial yeast and will both allow to evaluate the consequences of the introduced changes on a genomic scale and speed up the development of novel strains.

Wine yeast strains obtained by genetic engineering using recombinant methods are still perceived in a very controversial manner by consumers, are not likely to become commercially feasible and probably will not receive approval in the European Union within the next future. Further obstacles are complex legal and regulatory issues that require a detailed safety and environmental impact evaluation. Non-recombinant modification and optimization of industrial strains by whole cell engineering approaches or by "self-cloning", based on the use of host-derived genetic material are most likely to receive approval by both authorities and consumers.

The present chapter gives a global overview of recent advances regarding the importance and implications of the use of engineered *S. cerevisiae* strains in the wine industry, considering a variety of aspects such as the genetic constitution, ecology and population genetics of indigenous *S. cerevisiae* strains, phenotypes of interest in wine-emaking, strategies and targets used for the construction of the strains, taking also into account data derived from genomic and proteomic studies. The final part focuses on current legislation requirements and environmental risk evaluations concerning the deliberate release of GMY strains and includes an analysis of the reasons responsible for critical consumer's attitudes toward their application in winemaking.

The ecology and population genetics of *Saccharomyces cerevisiae*

Winemaking is a human activity for several millennia and the species *S. cerevisiae* can be considered as mankind's oldest domesticated organism (Pretorius, 2000). Molecular evidence of the historical presence of *S. cerevisiae* in wine fermentation has been obtained from identification of this species in pottery jars found in the tomb of one of the first Egyptian kings, which dates back to 3150 bc (Cavalieri *et al.*, 2003).

Traditional wine fermentation occurs in a spontaneous way when yeast, part of the indigenous microbial flora of the grape's surface, are brought in contact with the sugar-rich (20-30%) grape must, that is obtained from pressed crushed grapes. The composition of the grape's yeast flora depends on a large variety of factors such as climatic conditions including temperature and rainfalls, geographic localization of the vineyard (Parish and Carroll, 1985; Longo *et al.*, 1991), antifungal applications (Monteil *et al.*, 1986), soil type (Farris *et al.*, 1990), grape variety and the vineyard's age (Martini *et al.*, 1980; Rosini, 1982; Pretorius *et al.*, 1999). Predominant species on healthy grapes are apiculate yeasts like *Hanseniaspora uvarum* (and its anamorph form *Kloeckera apiculata*) and oxidative species such as *Candida*, *Pichia*, *Kluyveromyces* and *Rhodotorula* (Fleet and Heard, 1993). Fermentative species of the genus *Saccharomyces*, predominantly *S. cerevisiae*, occur in extremely low number on healthy undamaged grape berries (<0.1%) or in soils (Parish and Carroll, 1985; Frezier and Dubourdieu, 1992; Martini *et al.*, 1996), while damaged grapes are believed to be an important source, providing inocula of 10^2–10^3cells/ml of must (Mortimer and Polsinelli, 1999). Insects (e.g. *Drosophila* spp., honey bees and wasps), birds and wind are important agents for the dispersal of yeasts in habitats related to winemaking environments. Several ecological surveys, using molecular methods of identification, report a large diversity of genetic patterns among the enological fermentative flora. *S. cerevisiae* strains seem to be widely distributed in a given viticultural region, can be found in consecutive years and there are also predominant strains in the fermenting flora, hypothesizing the occurrence of specific native strains that can be associated with a *terroir* (Frezier and Dubourdieu, 1992; Vezinhet *et al.*, 1992; Versavaud *et al.*, 1995; Sabate *et al.*, 1998; van der Westhuizen *et al.*, 2000; Torija *et al.*, 2001; Lopes *et al.*, 2002; Schuller *et al.*, 2005; Valero *et al.*, 2007).

Independent studies report the prevalence of *S. cerevisiae* strains among the wineries resident flora (Longo *et al.*, 1991; Vaughan-Martini and Martini, 1995;Constanti *et al.*, 1997; Beltran *et al.*, 2002; Sabate *et al.*, 2002). This lead to the discussion whether the vineyard is a natural environment of *S. cerevisiae*, or just provides a source of "domesticated" isolates that passed through consecutive series of must fermentations and survived in the vineyard/winery until the following harvest and fermentation. The isolation of *S. cerevisiae* far from vineyards, for example from soils associated with oak trees in the north-eastern United States (Naumov *et al.*, 1998), oak exudates and other broad-leafed trees (Sniegowski *et al.*, 2002), but also from the Danube River (Slavikova and Vadkertiova, 1997) and the gut of insects supports a natural occurrence of this species in very diverse habitats.

When the yeast genome was fully sequenced, the community of yeast researchers has developed a keen interest in genetic variation of natural populations and its functional and evolutionary implications. One of the first population-genetic variation studies was undertaken by sequencing the four loci *CDC19*, *PHD1*, *FZF1* and *SSU1* in 27 *S. cerevisiae* strains. Sequence analysis of

each gene distinguished strains collected from a Pennsylvanian oak forest and strains collected from vineyards, perhaps due to ecological rather than geographic factors (Aa *et al.*, 2006). However, the finding of *S. cerevisiae* isolates in other sources still cannot exclude the prevailing idea that they simply represent migrants from fermentations and derived from a domesticated species, specialized for the fermentation of alcoholic beverages. A recent study showed that the species as a whole is not domesticated and consists of both ''wild'' and ''domesticated'' populations. In this study, genealogical relationships from DNA sequence diversity at five loci in 81 strains of *S. cerevisiae* isolated from fermentations, tree exudates and immuno-compromised patients were established. At least two independent domestication events lead to specialized breeds of *S. cerevisiae*, one for the production of grape wine and one for the production of sake wine. The oldest lineages and most of variation were found in strains from sources that are not related to wine production, suggesting that strains of *S. cerevisiae* specialized for the production of alcoholic beverages derived from natural populations unassociated with alcoholic beverage production, rather than the opposite (Fay and Benavides, 2005).

Parallel to the selection and development of new *S. cerevisiae* strains for enological applications, molecular methods were developed and validated to study the evolution of yeast flora in spontaneous, but also in inoculated fermentations. The most widely used typing methods are based on chromosome separation by pulsed field electrophoresis (Carle and Olson, 1985; Blondin and Vezinhet, 1988), restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) (Dubourdieu *et al.*, 1984; Vezinhet *et al.*, 1990; Querol *et al.*, 1992; Lopez *et al.*, 2001), randomly amplified polymorphic DNA, PCR fingerprinting followed by enzymatic restriction of amplified DNA (Baleiras Couto *et al.*, 1996), PCR-amplification of inter-delta sequences (Ness *et al.*, 1993, Legras and Karst, 2003) and multi locus sequence typing (MLST) (Ayoub *et al.*, 2006). In the last few years, fingerprinting of microsatellite or SSR (Simple Sequence Repeats) *loci*, short (1-10 nucleotides) DNA tandem repeats dispersed throughout the genome and with a high degree of variability, revealed to be very useful to discriminate *S. cerevisiae* strains (Gallego *et al.*, 1998; Hennequin *et al.*, 2001; Pérez *et al.*, 2001; Techera *et al.*, 2001; Schuller *et al.*, 2004). These loci exhibit a substantial level of polymorphism and have been used in humans for paternity tests, forensic medicine and population structure studies. Despite the higher equipment investment and need for skilled human resources, PCR-based microsatellite amplification and detection by capillary electrophoresis should be considered the method of choice, because of the easy high-throughput data generation, the absence of errors resulting from local experimental conditions and the possibility of sharing data by different laboratories. Besides the high level of discrimination and unequivocal results, expressed as base pair number (or as repeat number), the generated data are suitable to complete computational population genetic analysis. Twelve highly polymorphic micr-osatellite loci were used to assess the genetic diversity among 651 *S. cerevisiae* strains from 56 worldwide geographical origins. The genotypes clustered in

subgroups, according to the technological use (i.e. bread, beer, wine, sake). Bread strains displayed a combination of alleles intermediate between beer and wine strains, and strains used for rice wine and sake were most closely related to beer and bread strains. Macrogeographical differentiation of strains from Asia, Europe and Africa accounted for 28% of the observed genetic variation, which suggests clonal reproduction and local domestication of natural strains originating from the same geographic area. The data also indicated a Mesopotamia-based origin of most wine strains, and a migration route along the Danube Valley and around the Mediterranean Sea. The close association between vine migration and wine yeast favors the hypothesis that yeast may have followed man and vine as a commensal member of grapevine flora (Legras *et al.*, 2007). Similar phylogenetic relationships related to technological applications were observed when clustering of *S. cerevisiae* strain was based on 32 single-nucleotide polymorphism markers (Ben-Ari *et al.*, 2005) or amplified fragment length polymorphism (AFLP) analysis (Azumi and Goto-Yamamoto, 2001). Microsatellite revealed as informative markers for distinguishing populations from vineyards in very close geographical locations (50–100 km). Genetic differences among *S. cerevisiae* populations were rather apparent from gradations in allele frequencies than from distinctive "diagnostic" genotypes, and the accumulation of small allele-frequency differences across six loci allowed the identification of population structures. Within a vineyard, the genetic differentiation increased with the distance between sampling points suggesting a pattern of isolation by distance (Schuller and Casal, 2007).

Genetic constitution of *Saccharomyces cerevisiae* wine strains

When the *S. cerevisiae* genome sequencing project was completed, it became clear that this yeast has a genome of around 13000 kb, containing ca. 6000 protein-encoding genes that are distributed on 16 linear chromosomes, varying in length from 200 to 2200 kb, with a very low number of introns and little repetitive DNA (Goffeau *et al.*, 1996). Wild strains of *S. cerevisiae*, isolated from wine, cellars or vineyards are predominantly diploid, homothallic and mostly homozygous (65%), with low (Bakalinsky and Snow, 1990; Barre *et al.*, 1992; Guijo *et al.*, 1997) to high (>85%) sporulation capacity (Mortimer, 2000). Aneuploid strains, with approximately diploid DNA contents, have been described (Codon *et al.*, 1997; Nadal *et al.*, 1999; Puig *et al.*, 2000) and meiosis seems not to be a common occurrence in their life-cycle (Bakalinsky and Snow, 1990; Barre *et al.*, 1992). Such wine yeast strains present essentially an asexual life cycle and are characterized by high karyotype instability, which is believed to be a potential source of genetic variability (Bidenne *et al.*, 1992; Longo and Vezinhet 1993; Nadal *et al.*, 1999; Carro *et al.*, 2003). Haploid laboratory strains do not undergo by far such extensive changes (Longo and Vezinhet, 1993).

Gross mitotic chromosomal rearrangements, such as large regions fusion between homologous and non-homologous chromosomes occur in wine yeast with frequencies around 10^{-5} (Puig *et al.*, 2000). In chromosome I, several

membrane-associated genes are located in subtelomeric regions, and it was hypothesized that subtelomeric plasticity may allow rapid adaptive changes of the yeast strain to specific substrates (Carro *et al.*, 2003). The *SSU1-R* allele, generated by reciprocal translocation between chromosomes VIII and XVI, confers sulfite resistance to yeast cells and was described as the first case of adaptive evolution, occurring probably because sulfite was used as a preservative in wine production (Goto-Yamamoto *et al.*, 1998; Pérez-Ortin *et al.*, 2002). Retrotransposons may also be involved in chromosomal recombinations. *S. cerevisiae* strains contain between two and 30 copies of at least five retrotransposons (Ty1-Ty5), being the copy number of each highly variable, depending on the strain examined. Multiple Ty elements mediated reciprocal recombinations (chromosome I/III or III/VII) were shown by fine-mapping of the junctions, demonstrating their crucial involvement in karyotype alterations in natural and industrial strains (Rachidi *et al.*, 1999; Umezu *et al.*, 2002; Carro *et al.*, 2003), together with insertions/ transpositions of Y′elements (Neuvéglise *et al.*, 2000).

Among *Saccharomyces* yeasts used in wine, beer and cider production, genetically stable interspecies hybrids, that possesses nuclear DNA from two or three species are quite common. The strain CID1, which was isolated from a home fabricated apple cider (Masneuf *et al.*, 1998) is a triple hybrid between *S. cerevisiae*, *S. kudriavzevii*, and *S. bayanus* var. *uvarum*, as was shown by analysis of the partial sequence of the *ACT1* gene, flow cytometry analysis (Naumova *et al.*, 2005a) and by amplified fragment length polymorphism analysis (de Barros Lopes *et al.*, 2002). *S. cerevisiae* x *S. bayanus* var. *uvarum* hybrids were also identified among baker's yeast and hybrids were also obtained from the surface of black-currant berries (Naumova *et al.*, 2005b). *S. cerevisiae* x *S. bayanus* var. *uvarum* diploid hybrids were isolated from spontaneous fermentations and microsatellite DNA analysis identified strains isolated in the same cellar as potential parents belonging to *S. bayanus* var. *uvarum* and *S. cerevisiae*. Such genetic mixes may be useful from a technological standpoint because they lead to the emergence of more vigorous, competitive strains, combining the specific properties of the parental strains (le Jeune *et al.*, 2007). *S. cerevisiae* x *S. kudriavzevii* hybrids can also be involved in wine fermentation, as was shown by sequence analysis of the mitochondrial gene *COX2* and restriction analysis of nuclear and ribosomal genes (5.8S rRNA) (Gonzalez *et al.*, 2006).

The advent of DNA microarray technology has enabled the analysis of global patterns of gene expression and diverse networks of coordinated function. However, the genetic differences examined have been primarily differences between growth conditions or between mutant strains and this knowledge has accumulated on a narrow range of laboratory yeast genetic backgrounds, selected due to their suitability to laboratory conditions. In the last few years, genetic variation among laboratory, but also natural isolates, became unravelled on a genomic scale. The studies, summarized as follows, revealed considerable genetic divergence among *S. cerevisiae* strains.

Natural vineyard populations of *S. cerevisiae* harbor alleles that cause massive alterations in gene expression as was shown by combining classical Mendelian segregation analysis with microarray-based genomics. The four progeny of a natural isolate (M28) from Tuscany segregated 2:2 for filagree and smooth colony phenotypes. In cultures derived from middle-logarithmic phase in YPD medium (yeast extract 1% w/v, peptone 2% w/v, and glucose 2% w/v), almost 400 genes, mostly associated with amino acid biosynthesis and transport, sulphur or nitrogen assimilation were differentially expressed between the two phenotypes. The filagree progeny poorly express genes for amino acid transport and instead abundantly express genes for the synthesis of amino acids. Differentially expressed genes segregated as a suite of traits, due to variation in a few regulatory loci that either act on hundreds of loci or initiate cascades of transcriptional control. These studies showed that natural vineyard populations of *S. cerevisiae* can harbor alleles that cause massive alterations in the global patterns of gene expression (Cavalieri *et al.*, 2000). Under the same experimental conditions, another study examined gene-expression variation of the M28 strain to three other isolates from the same set of vineyards around Montalcino, Italy. Among the four isolates, 433 genes were expressed at significantly different levels between at least two isolates, and most variation was found in genes associated with amino acid metabolism, protein synthesis and degradation, metal ion transport and transposable element activity (Townsend *et al.*, 2003). The commercial wine yeast strain T73 and the laboratory strain S288C showed significant differential expression patterns in 40 genes during logarithmic growth in YPD medium. These genes were mainly associated with small changes in promoter regions or variations in gene copy number (Hauser *et al.*, 2001).

DNA-array-based hybridisation is an emerging and powerful method for scanning genomes that allows for genome-wide genotyping. By commercial high-density oligonucleotide arrays that contain up to 200 000 different 25 mers features from the yeast genomic sequence genome-wide diversity between strains can be determined with a level of detail previously impossible. Single-base pair changes between two 25 bp sequences, especially in the central zone, can disrupt hybridization. They are used to determine the genetic variation (locations of allelic differences) existing between two strains and whether functional classes of genes or particular genome regions show higher rates of variability. One of the first large-scale studies to discover variable genes within *S. cerevisiae* populations was published by Winzeler *et al.* (2003). Using 14 yeast strains, common laboratory strains and natural isolates, it was shown that intra-species genome variability is biased toward subtelomeric regions at the ends of chromosomes, where genes related to fermentation and transport are located (Winzeler *et al.*, 2003). This approach will be fundamental for future genome evolution and population genetic studies in yeast, but has also great potential for the rapid identification of loci that are responsible for imparting positive attributes.

Unexpectedly wide differences exist even when comparing laboratory strains. The popular laboratory strains S288C and CEN.PK113-7D showed significant physiological differences in protein expression and lipid metabolism. Comparison by high-density oligonucleotide arrays revealed divergent hybridization patterns in 288 genes, due to differential amplification, gene absence or sequence polymorphisms. Seventeen genes were absent in CEN.PK113-7D and eight genes did not show hybridization signals due to significant differences at the DNA level compared to S288C (Daran-Lapujade *et al.*, 2003).

A global view of genetic variation among commercial wine strains both at intra- and inter- strain level has been obtained by microarray karyotyping, also known as "arraycomparative genomic hybridization" ("aCGH"), giving information on whole or partial chromosome aneuploidies, non-reciprocal translocations and isolated gene deletions or amplifications by the examination of copy number changes for every gene. The analysis of three independent isolates of each of four commonly used commercial *S. cerevisiae* wine strains relative to each other and to the sequenced *S. cerevisiae* strain S288C, showed that a major group of shared genomic differences, found among all wine strains, is associated with genes coding for membrane transporters or genes involved in drug resistance pathways (Dunn *et al.*, 2005). The low level of inter-strain variability suggests that it can be relatively easy to discover whether the observed differences do indeed confer different sensory properties in the finished wine, but differences in the fermentation and organoleptic properties may also arise from single nucleotide changes, of which there may be many, and that are not detected by microarray karyotyping.

Infante *et al.* (2003) performed a detailed microarray karyotyping study of the genomic differences between two *S. cerevisiae* "flor" yeasts, obtained from the velum, a unique biofilm, which develops on the surface of the wine during the sherry wine making. The strains differed from one another in genomic regions that are flanked by repeated sequences or other recombination hotspots and that could mediate the observed chromosomal rearrangements by nonallelic interactions. However, the authors only compared the two "flor" yeasts to each other, and not to the sequenced S288C laboratory strain (Infante *et al.*, 2003).

Selection of *Saccharomyces cerevisiae* strains with desirable characteristics

S. cerevisiae populations harbor genetic variation that is associated with geographical ecological factors and that is the basis of the well-described phenotypic variability that has been explored for decades in strain selection programs. It is consensual among winemakers that the choice of wine yeast strain has a major impact on the sensory characteristics of both still and sparkling wines. Selection for millennia of wine-making may have created unique and interesting oenological traits, but they are not widely distributed, nor can be found in combination in one strain. Clonal selection of wild *Saccharomyces* strains isolated from natural environments belonging to the viticultural areas of

interest is always the starting point for a wine yeast selection program. It is desirable to evaluate the phenotypic diversity for as much as possible traits, such as glycerol production (Remize *et al.*, 2000b), hydrogen sulphite formation (Mendes-Ferreira *et al.*, 2002) or the modulation of grape-derived volatile thiols such as 4-mercapto-4-methylpentan-2-one (4MMP) during wine fermentation (Howell *et al.*, 2004). Currently, about 150 different wine yeast strains, mainly *S. cerevisiae*, are commercially available as active dry yeast, and are widely used due to their superior oenological properties, contributing to both standardization of fermentative processes and wine quality. Contrarily, spontaneous fermentations are usually used by small boutique wineries that wish to emphasize vintage variability, reflecting the specificity of a particular region, and that rely merely on indigenous yeasts present on the grape skin, which are thought to produce wines with a fuller palate structure. The first commercialized wine yeast strains were simply expected to ensure complete fermentation with rapid kinetics, but the criteria have evolved over the years, since the particular strain used should be most suitable for each type of wine to be produced. The current trend toward the production of high quality wines with distinctive and very characteristic properties requires the use of "special yeasts for special traits" (Pretorius, 2000; Mannazzu *et al.*, 2002; Romano *et al.*, 2003b).

Definition of the appropriate selection strategy should always depend on the traits that a wine strain is supposed to harbor and the number of strains to be screened. As summarized in Table 1.1, numerous oenological characteristics were proposed to be evaluated. Technologically relevant data can be obtained by monitoring the fermentation progress, and quantitative traits are determined by chemical analysis at the end of fermentation.

Table 1.1. Oenological characteristics considered in the selection of *S. cerevisiae* wine strains (Regodon *et al.*, 1997; Romano *et al.*, 1998; Guerra *et al.*, 1999; Maifreni *et al.*, 1999; Perez-Coello *et al.*, 1999; Esteve-Zarzoso *et al.*, 2000; Rainieri and Pretorius 2000; Steger and Lambrechts, 2000; Martinez-Rodriguez *et al.*, 2001; Brandolini *et al.*, 2002; Caridi *et al.*, 2002; Mannazzu *et al.*, 2002; Mendes-Ferreira *et al.*, 2002).

Oenological characteristics	Comments
Fermentation vigor	Maximum amount of ethanol (%, v/v) produced at the end of the fermentation; Desirable: good ethanol production
Fermentation rate	Grams of CO_2 produced during the first 48 hours of fermentation Desirable: prompt fermentation initiation
Mode of growth in liquid medium	Dispersed or flocculent growth, sedimentation speed Desirable: dispersed yeast growth during, but sedimentation at the end of fermentation
Foam production	Height of foam produced during fermentation Undesirable: increased foam production

Optimum fermentation temperature	Thermotolerance and cryotolerance is related to oenological properties Optimum fermentation temperature ranges between 18 and 28°C
Volatile acidity, acetic acid production	Selected strains should not release more than 100 – 400 mg l^{-1} during fermentation Undesirable: increased volatile acidity/acetic acid production
Malic acid degradation or production	Whether degradation of production is desirable depends on the characteristics of the must. Malic acid degradation varies between 0-20% depending on the *S. cerevisiae* strain
Glycerol production	Desirable major fermentation by-product (5-8 g l^{-1}) contributing to wine sweetness, body and fullness
Acetaldehyde production	Desirable metabolite in sherry, dessert and port wines being an important character for selection of strains to be applied in wine ageing
Esters, higher alcohols and volatile compounds	Desirable metabolites, markedly influence wine flavor and depend on the presence of precursors related to both grape cultivar and grape maturity. Limited amounts contribute positively to global sensorial characteristics
SO_2 tolerance and production	Antioxidant and antimicrobial agent Desirable: high fermentation vigor and rate in the presence of SO_2 concentrations usually applied in winemaking; Undesirable: excessive SO_2 production
H_2S production	Determined as the strains colony color on a bismuth containing indicator medium, e.g. BIGGY Agar; H_2S is detrimental to wine quality, considered as off-flavor with very low threshold value (50-80 µg/l)
Stress resistance	Tolerance to combined acid/osmotic stress
Copper resistance	High copper concentrations may cause stuck fermentations Desirable: high copper resistance and the ability to reduce the copper content

As mentioned in the previous section, recent research has provided interesting findings of naturally occurring *Saccharomyces* hybrid strains, for example triple hybrids S. *cerevisiae*, x *S. bayanus* x *S. kudriavzevii* (Gonzalez *et al.*, 2006). *S. cerevisiae* × *S. kudriavzevii* hybrids were found to have a promising enological potential, since they were better adapted to alcoholic fermentations carried out at lower temperatures (14-22°C), produced less acetic acid and intermediate amounts of glycerol in combination with increased amounts of higher alcohols (Gonzalez *et al.*, 2007). Strains with improved technological properties were also obtained from hybrids between cryotolerant *S. bayanus* and thermotolerant *S. cerevisiae* strains (Rainieri *et al.*, 1998; Masneuf *et al.*, 2002; Coloretti *et al.*, 2006).

Finding wine yeast strains possessing an ideal combination of oenological characteristics is highly improbable and therefore selection programs were extended to non-*Saccharomyces* species, e.g. *Candida*, *Kloeckera*, *Debaryo-*

myces, *Hanseniaspora*, *Hansenula*, *Pichia*, *Metschnikowia*, *Schizosaccharomyces*, *Saccharomycodes* or *Rhodotorula*. Although non-*Saccharomyces* species lack competitiveness in oenological conditions mainly because they are not vigorously fermenting and display a lower stress resistance when compared to *S. cerevisiae*, the use of mixed starter cultures or sequential fermentation (e.g. *C. cantarellii*/ *S. cerevisiae*) for directing fermentations toward enhanced glycerol and reduced acetic acid production has been successfully used (Toro and Vazquez, 2002). The yeasts *Torulaspora delbrueckii* and *Candida stellata* are considered to be positive contributors to the overall organoleptic wine characteristics, while apiculate yeasts such as *Kloeckera apiculata* have a negative influence on wine quality due to pronounced acetic acid and ethyl acetate formation associated with low ethanol production (Ciani and Maccarelli, 1998).

Countless references report the beneficial and detrimental influence of non-*Saccharomyces* yeasts on the volatile composition of musts from varying grape varieties (Ciani and Maccarelli, 1998; Granchi *et al.*, 2002; Mingorance-Cazorla *et al.*, 2003; Plata *et al.*, 2003; Romano *et al.*, 2003c; Clemente -Jimenez *et al.*, 2004), and considerable differences regarding these compounds were also found among commercial or autochthonous *S. cerevisiae* strains (Steger and Lambrechts, 2000; Patel and Shibamoto, 2003; Romano *et al.*, 2003a). Non-*Saccharomyces* yeasts, especially selected and commercialized for aroma and flavor enhancement in wine, for example as a blend of *S. cerevisiae* / *Kluyveromyces thermotolerans* / *Torulaspora delbrueckii* or *S. cerevisiae* / *Kluyveromyces thermotolerans*. Immobilized *Schizosaccharomyces pombe* cells are also commercially available for the biological reduction of wine acidity by malic acid consumption (Silva *et al.*, 2003).

Non-*Saccharomyces* strains metabolize grape-derived precursor compounds, contributing thus to reveal the varietal aroma and improve the winemaking process (Fleet and Heard, 1993; Esteve-Zarzoso *et al.*, 1998; Fernandez *et al.*, 2000; Otero *et al.*, 2003). Pectinases increase juice extraction, improve clarification and facilitate wine filtration, ß-glycosidases hydrolyse non-volatile glycosidic aromatic precursors from the grape, proteases improve the clarification process, esterases contribute to aroma compound formation and lipases degrade lipids from grape or yeast autolytic reactions. *S. cerevisiae* is not a significant producer of such enzymes with relevance in wine production, being mainly ß-glycosidase production reported for this species (Restuccia *et al.*, 2002; Rodriguez *et al.*, 2004).

Albeit the high amount of phenotypic variation that can be found among *S. cerevisiae* strains, and the inclusion of non-*Saccharomyces* and hybrid starter strains with a whole range of specialized properties that can add value to the final product, there is no doubt that significant progress of technological, fermentative and aromatic characteristics can only be achieved via targeted breeding and genetic engineering programs.

S. cerevisiae strain modification based on classical methods

Wine yeast strains exhibit a wide variability in their biotechnological properties, and the genetic diversity of native isolates has provided ample material from which to select wine yeasts expressing specific traits. In fact, the vast majority of *S. cerevisiae* strains currently on the market derived from isolation and screening of strains obtained from wineries or from vineyards. However, the natural availability of strains possessing an ideal combination of oenological characteristics is highly improbable because the most important enological traits, such as ethanol tolerance, low volatile acidity production or hydrogen sulphide production, are polygenic features, with complex interactions between alleles. A population of 50 progeny clones derived from four industrial wine strains of *S. cerevisiae* demonstrated that many clones presented better aptitudes than the parental strains in regard to ethanol tolerance, volatile acidity and hydrogen sulphide production, and that traits are in part inheritable and clearly polygenic (Marullo *et al.*, 2004).

Classical methods for strain modifications include mutagenesis or hybridization, where large genomic regions or entire genomes are recombined or rearranged. Elimination of undesirable characteristics and enhancement of favorable properties has been addressed through mutagenesis, using UV radiation or chemical agents such as ethylmethane sulfonate due to the low average spontaneous mutation frequency in yeast populations. A drawback of such methods is the effect of ploidy, which reduces efficiency in diploid or polyploid strains, and the presence of non-mutated alleles that cannot be easily detected. Therefore, haploid strains are preferred when inducing mutations, and mutagenesis is usually applied to isolate new variants of wine yeast strains before further genetic manipulation. A mutant wine strain was obtained by UV-mutagenesis, carrying a recessive allele of *ure2* that deregulated the proline utilization pathway, characterized by abolished nitrogen catabolite repression through ammonium ions. The strain showed an improved fermentation performance in media where proline and other poorly assimilated amino acids are the major potential nitrogen source, as is the case for most fruit juices and grape musts (Salmon and Barre, 1998). Mutants with an accelerated autolysis during second fermentation of sparkling wines were also obtained. This process is associated with the release of intracellular compounds that modify the chemical composition and sensory properties and usually lasts from a few months to years (Gonzalez *et al.*, 2003; Nunez *et al.*, 2005). UV mutagenesis was also used to obtain a thermosensitive autolytic mutant affected in cell wall integrity, with improved capability to release cell wall mannoproteins during alcoholic fermentation (Giovani and Rosi, 2007). Such polysaccharides confer greater body and smoothness to white and red wines (Vidal *et al.*, 2004).

Modulating a specific property can be easily achieved by intra-specific hybridization, based on the sexual cycle of *S. cerevisiae*, where a new heterozygous diploid cell is produced by sporulating parental diploids, recovering individual haploid ascospores and mating haploid progeny of opposite mating

types. Several hybrid strains are currently on the market, and this approach is still considered the most effective method for improving and combining traits under polygenic control, particularly if the molecular nature of the mechanisms involved has not been elucidated. Intra-specific hybridization was used, for example, to eliminate undesirable properties like SO_2 formation or excess foaming (Eschenbruch *et al.*, 1982). A flocculent *S. cerevisiae* strain to be used in the production of sparkling wines and not producing H_2S was obtained by hybridizing a flocculent strain with a H_2S non-producing strain (Romano *et al.*, 1985). Classical sexual reproduction has proven difficult for the case of homothallic strains, in which mating type reversals and cell fusion/diploid formation occurs in a spontaneous way. In this situation, particular forms of hybridization can be applied, such as spore-cell mating in which homothallic ascospores from the same ascus are placed into direct contact with heterothallic haploid cells. Spore-cell mating was used for the optimization of 11 relevant enological traits, by crossing two strains derived from commercial wine strains, a homozygous heterothallic strain carrying the *ho::KanMX*4 allele with the ascospores of a homothallic strain. In an additional targeted sexual cross, from the segregating progeny, all the optimal characters from both parents were combined in a single strain, showing the usefulness of this method for obtaining a wine strain with numerous fermentative qualities (Marullo *et al.*, 2006).

There are other forms of hybridization such as protoplast fusion, which is a direct, asexual technique that can be used to fuse non-sporulating yeast strains, surpassing the natural barriers of hybridization. Desirable (and undesirable) characteristics of both parental strains will recombine in the offspring. This approach can also be used to fuse cells with different levels of ploidy. Triploid strains can be obtained by fusion of a diploid to a haploid strain.

One of the limitations when hybridizing strains belonging to the same species is that traits to be exchanged or introduced in the hybrid culture and in its progeny are limited to the species-specific characteristics. Recent genetic analysis showed that there are no barriers to interspecific conjugation among *Saccharomyces sensu stricto* yeasts (Masneuf *et al.*, 1998;de Barros Lopes *et al.*, 2002;Naumova *et al.*, 2005a; Naumova *et al.*, 2005b;Gonzalez *et al.*, 2006), and that introgression may lead to the emergence of more vigorous, competitive strains, combining the specific properties of the parental strains (Coloretti *et al.*, 2006; le Jeune *et al.*, 2007). *S. bayanus*, for example, is a cryotolerant species and has a better fermentative profile at low temperatures compared to *S. cerevisiae* (Kishimoto and Goto, 1995). Wines fermented by *S. bayanus* are characterized by smaller amounts of acetic acid and ethanol, higher amounts of glycerol, succinic and malic acid (Kishimoto *et al.*, 1993; Zambonelli *et al.*, 1997). Besides, this species produces wines with higher amounts of flavor-active esters, such as β-phenylethyl alcohol and β-phenylethyl acetate (Masneuf *et al.*, 1998). Hybrids of *S. cerevisiae* x *S. bayanus* obtained in the laboratory exhibited such characteristics at midway the parental strains. This can present an advantage in wine making, especially for white wines, which are fermented at a low

temperature and for which intermediate amounts of β-phenylethyl alcohol and its acetate are desirable.

The previously mentioned methods of strain modification by mutagenesis or hybridization are used to improve and combine traits under polygenic control, but the introduced genetic changes remain hidden. Since they rely on classical breeding methods by which large genomic regions or entire genomes are recombined or rearranged, the resulting strains are not considered as GMOs according current legislative definitions.

Targets for genetic modifications by recombinant DNA technologies

Genetic improvement of industrial strains by classical genetics was followed in the last 20 years by the use of recombinant DNA technologies that made the construction of specialized commercial strains possible, mainly by heterologous gene expression or by manipulation of a specific metabolic pathway associated with altered gene dosage by modification of the gene promoter. Recombinant strain construction is easy and feasible, as far as the desired trait is encoded by one or few well-characterized genes.

The most important targets for wine strain improvement are related to higher ethanol tolerance, enhanced wholesomeness and organoleptical properties through altered sensorial characteristics, and improvements for processing efficiency (Blondin and Dequin, 1998; Pretorius, 2000; Dequin, 2001; Pretorius and Bauer, 2002; Dequin *et al.*, 2003; Pretorius *et al.*, 2003; Marullo *et al.*, 2004; Marullo *et al.*, 2006; Verstrepen *et al.*, 2006). Table 1.2 shows examples of the way in which *S. cerevisiae* wine yeast strains are currently being developed.

The worldwide growing demand for wines containing lower levels of alcohol has been addressed by engineering wine yeast strains that produce lower amounts of ethanol during alcoholic fermentation. This issue has been addressed by integration of the *Aspergillus niger* glucose oxidase *GOX1* gene into the *S. cerevisiae* genome. Wines produced with this yeast had 1.8–2.0% less alcohol, which was ascribed to production of d-glucono-δ-lactone and gluconic acid from glucose by *GOX* (Malherbe *et al.*, 2003). Efficient decrease (15–20%) of ethanol yield was succeeded through metabolic re-routing of glucose toward glycerol through overexpression of *GPD1*, encoding glycerol-3-phosphate dehydrogenase, combined with *ALD6* deletion, encoding acetaldehyde dehydrogenase to abolish excessive acetate production as a major side effect. However, this strain accumulated acetoin, which has a negative sensorial impact on wine (Cambon *et al.*, 2006). An alternative strategy was based on constitutive expression of an H_2O-NADH oxidase from *Lactoccocus lactis* in *S. cerevisiae*. However, the marked decrease in the intracellular NADH pool lead to reduced growth and fermentative performance (Heux *et al.*, 2006a), that could be improved when the anaerobic growth phase was followed by a microaeration phase with nongrowing cells. This strain reduced ethanol yield by 7%, but still showed a specific metabolite redistribution pattern, characterized by the presence of undesirable

oxidized metabolites such as acetaldehyde, acetate and acetoin (Heux *et al.*, 2006b).

The aromatic profile of a wine comprises hundreds of compounds that are interacting in a highly complex manner. Well-balanced wines must evidence characteristic flavor and aroma notes, whereas undesirable flavor compounds and metabolites should be absent. Metabolic yeast metabolites such as esters or alcohols contribute to the complexity and intensity of the final wine. Numerous approaches have been undertaken to develop *S. cerevisiae* starter strains that could impart specific desirable aromas and flavors by constitutive expression or overexpression of enzymes such as endoglucanase, arabinofuranosidase, endoxylanase or rhamnosidase for the cleavage of aroma components from their glycosylated precursors, producing wines with an increased fruity aroma (Pérez-González *et al.*, 1993; Sanchez-Torres *et al.*, 1996; Ganga *et al.*, 1999; Manzanares *et al.*, 2003). Starter strains have been constructed with optimized decarboxylation activity of phenolic acids, resulting in volatile phenols such as 4-vinyl and 4-ethyl derivatives that positively influence wine aroma (Smit *et al.*, 2003). Cysteinylated thiols are grape-derived non-volatile precursors of volatile thiols, that enhance the varietal characters and impart flavors of passionfruit, grapefruit, gooseberry, blackcurrant, lychee, guava and box hedge. A *S. cerevisiae* strain expressing tryptophanase with strong cysteine-beta-lyase activity released up to 25 times more volatile thiols and the produced wines displayed an intense passionfruit aroma (Swiegers *et al.*, 2007). Some of the most important yeast-derived aroma compounds produced are esters such as ethyl acetate and isoamyl acetate. Volatile esters represent the largest and most important group of flavor compounds produced during fermentation, and C_4–C_{10} fatty acid ethyl esters confer characteristic fruity odors such as apple-like (hexyl acetate, ethyl caproate and ethyl caprylate) or banana-like (isoamyl acetate) (Swiegers *et al.*, 2005). Recent approaches have been undertaken investigating the interactive roles of ester-synthesizing and ester-hydrolyzing enzymes in wine yeast to develop strains with differential ester-producing capabilities that could assist winemakers in their effort to consistently produce wines according to definable flavor specifications and styles. Overexpression of the *ATF1* gene encoding alcohol acetyltransferases significantly increased the concentrations of ethyl acetate, isoamyl acetate, 2-phenylethyl acetate and ethyl caproate, that were efficiently degraded by the the *IAH1*-encoded esterase. *EHT1*-encoded ethanol hexanoyl transferase overexpression resulted in a marked increase in ethyl caproate, ethyl caprylate and ethyl caprate (Lilly *et al.*, 2006a). Manipulation of the intracellular pool of acetyl-CoA was shown to play a role in the development of ester aromas such as ethyl acetate and isoamyl acetate (Cordente *et al.*, 2007). Branched chain amino acids are the precursors for the biosynthesis of higher alcohols, also known as fusel alcohols. They are quantitatively the largest group of aroma compounds in wines. Lilly *et al.* (2006b) showed that constitutive expression of the branched-chain amino acid transaminase *BAT1* and *BAT2* facilitates the production of optimized concentrations of higher alcohols during wine fermentations.

Glycerol has no aromatic characteristics but rather contributes to the sensory character of wine by its sweet taste, and is quantitatively the most important fermentation product after ethanol and carbon dioxide. A 1.5- to 2.5-fold increase in glycerol production and a slight decrease in ethanol formation under conditions simulating wine fermentation was achieved by overexpression of the *GPD1* gene, encoding a glycerol-3-phosphate dehydrogenase. However, the resultant change in redox balance caused excessive formation of secondary metabolites such as succinate, acetate, acetoin and 2,3-butanediol (Michnick *et al.*, 1997; Remize *et al.*, 1999).

L-tartaric and L-malic acid are the predominant organic acids in wine and represent 70–90% of total grape acidity. Flavor problems associated to insufficient or excessive acidity may occur in wines produced in climatic hot or colder regions, respectively. Efficient biological acidity correction is of biotechnological interest to produce a high-quality wine with a fine balance between the sugar and the acid content. *S. cerevisiae* strains degrade malic acid in must only partially (10-25%) during alcoholic fermentation by the mitochondrial malic enzyme (Riberéau-Gayon *et al.*, 2000), and the capacity to use malic acid varies among *S. cerevisiae* strains (Subden *et al.*, 1998). The commercial strain Lalvin71B is promoted as malate degrading strain, but may have a variable performance, depending on the must type (our unpublished results). Genetically modified *S. cerevisiae* strains have been constructed by coexpression of the malate permease from *Schizosaccharomyces pombe* and the *mleS* malolactic gene from *Lactococcus lactis* (Bony *et al.*, 1997; Volschenk *et al.*, 1997a; Volschenk *et al.*, 2001) or the *mleA* malolactic gene from *Oenococcus oeni* (Husnik *et al.*, 2006).

Acetate is the main component of volatile acidity and plays a significant role in the organoleptic balance of wine. In wine, acetic acid is a by-product of yeast alcoholic fermentation, and is highly undesirable above the threshold of 0.8 g/l. A substantial decrease (40-75%) in acetate yield was achieved by *ALD6* gene disruption, encoding acetaldehyde dehydrogenase (Remize *et al.*, 2000a). Engineered *S. cerevisiae* strains over-expressing a bacterial lacticodehydrogenase (*LDH*) have been described to perform mixed lactic acid-alcoholic fermentation under enological conditions and increased total acidity by 50% through production of 5 g/L of L(+) lactic acid (Dequin *et al.*, 1999).

Yeast can also be responsible for the production of unwanted byproducts, such as hydrogen sulphide, that is synthesized during alcoholic fermentation. A promising strategy was designed for reducing hydrogen sulfide production, based on site-directed mutagenesis to lower the activity of NADPH-dependent sulfite reductase, a key enzyme in the biosynthesis of sulfur-containing amino acids (Sutherland *et al.*, 2003).

Novel wine yeast strains have been developed that could contribute to improved health-protective effects by increased resveratol formation, a health-promoting stilbene that is mainly formed in the grape skin. This compound is possibly associated with the "French paradox", i.e. a lower heart disease

incidence among the French population, where a high-fat diet is combined with the regular consumption of wine. Resveratrol synthesis was engineered by co-expression of the grapevine stilbene ligase gene (*VST1*) and co-enzyme A ligase encoding gene (*4CL216*) from hybrid poplar. Resveratrol production occurred from the synthesized p-coumaroyl-CoA and the yeast-derived 3-malonyl-CoA by stilbene ligase (Becker, *et al.*, 2003). Resveratrol content of white wine has also been increased by expression of *Aspergillus niger abfB* gene encoding an alpha-L-arabinofuranosidase or *Candida molischiana bglN* gene encoding a beta-glucosidase to increase free resveratrol from its glycosylated precursors (Gonzalez-Candelas *et al.*, 2000).

Focusing on health aspects, yeasts were developed that could minimize the risks associated with moderate wine consumption by elimination of ethyl carbamate, a suspected carcinogen that is sometimes formed in wine through spontaneous reaction of ethanol with urea, which is secreted by yeast cells. Under fermentative conditions in the presence of nitrogen sources, urea catabolism to ammonia by urea amidolyase, the product of the *DUR1,2* gene, is transcriptionally repressed. When *DUR1,2* was constitutively expressed, ethyl carbamate could be reduced by 89.1% (Coulon *et al.*, 2006).

The physicochemical characteristics and sensory properties of wine can be altered by undesired bacterial growth before, during or after fermentation. In general, growth control of unwanted microbial contaminants is provided by the addition of chemical preservatives such as sulphur dioxide or other antibacterial compounds and enzymes. The expression of antimicrobial enzymes and peptides in starter strains has been achieved by distinct approaches. Bactericidal yeasts, engineered by expressing genes encoding *Pediococcus acidilactici* pediocin (*PED1*) (Schoeman *et al.*, 1999) and *Leuconostoc carnosum* leucocin (*LCA1*) (du Toit and Pretorius, 2000) have been used to obtain bactericidal yeasts. The antifungal *CTS1*-encoded chitinase has also been expressed in *S. cerevisiae* (Carstens *et al.*, 2003), as well as the *GOX1*-encoded exoglucanase to inhibit wine spoilage organisms, such as acetic acid bacteria and lactic acid bacteria during fermentation (Malherbe *et al.*, 2003).

Clarification and physicochemical stability of wines is usually achieved by an increasing spectrum of relatively expensive commercial polysaccharase enzyme preparations, due to the inability of indigenous *S. cerevisiae* strains to degrade grape-derived polysaccharides such as glucan and xylan. Recombinant strains were obtained, by integrating the *Trichoderma reesei XYN2* xylanase gene construct and the *Butyrivibrio fibrisolvens END1* glucanase gene cassette into the genome of a commercial wine yeast strain. Wines obtained with the polysaccharide-degrading strains resulted in significant improvements in juice extraction, colour intensity and stability, as well as alterations in the aromatic profiles (Louw *et al.*, 2006). Pectinolytic *S. cerevisiae* strains have been constructed by expressing enzymes of fungal origin (Gonzalez-Candelas *et al.*, 1995) or polygalacturonase encoded by *PGU1* as an alternative to commercial enzyme preparations (Vilanova *et al.*, 2000). Proficient clarification at the end of

fermentation can also be achieved by regulated expression of the flocculation genes to guarantee efficient settling at the end of fermentation (Verstrepen *et al.*, 2001; Verstrepen *et al.*, 2006).

Strategies for genetic modifications

In general, all genetic material used for the construction of microorganisms used for food fermentation should be derived from the host species (self-cloning) or GRAS (generally regarded as safe) organisms with a history of safe food use. The use of DNA sequences from species taxonomically closely related to pathogenic species has to be avoided. Heterologous gene expression was used in most cases, being the genes of interest isolated for example from *Lactobacillus casei* (*LDH*), *Lactobacillus plantarum* (*pdc*), *Lactobacillus lactis* (*noxE*, *mleS*), *Leuconostoc carnosum* (*LCA1*), *Oenococcus oeni* (*mleA*), *Bacillus subtilis* (*padc*), *Pediococcus acidilactici* (*pedA*), *Fusarium solani* (*pelA*), *Trichoderma reesei* (*XYN2*), *Butyrivibrio fibrisolvens (END1), Erwinia chrysanthemi (PEL5), Erwinia carotovora (PEH1), Candida molischiana* (*bglN*), *Schizosaccharomyces pombe* (*mae1* and *mae2*), hybrid poplar (*4CL216*), grapevine (*vst1*), *Aspergillus* sp. (*GOX egl1*, *abfB*, *xlnA*, *rhaA*), *E.coli (tnaA)* or *Fusarium solani* (*pelA*), being others, such as *ATF1*, *GPD1* or *PGU1* derived from *S. cerevisiae* (Table 1.2).

Table 1.2. Targets for *S. cerevisiae* strain improvement, indicating, whenever possible, examples of the strategies used for genetic modifications

Modification	Protein(s)	Gene(s)	Source	Construction					Reference
				P	T	Pla	M	Chr	
Reduce ethanol content	Glucose oxidase	*gox*	*A. niger*	*PGH1*	*PGK1*		*URA3*	+	(Malherbe *et al.*, 2003)
	Glycerol-3-phosphate dehydrogenase	*GPD1*	*S. cevevisiae*	*ADH1*	*ADH1*	2μ	Tn5 *ble*	-	(Cambon *et al.*, 2006)
	Acetaldehyde dehydrogenase	*ALD6* deletion	*S. cevevisiae*				*kanMX4*		
	NADH oxidase	*noxE*	*L. lactis*	*TDH3*			*URA3*	+	(Heux *et al.*, 2006a; Heux *et al.*, 2006b)
Cleavage of aroma components from their glycosylated precursors	Endoglucanase	*egl1*	*T. longibrachiatum*	*ACT*	-	2μ	*CYH2*	-	(Pérez-González *et al.*, 1993)
	Arabinofuranosidase	*abfB*	*A. niger*	*ACT*	-	2μ	*CYH2*	-	(Sanchez-Torres *et al.*, 1996)
	Endoxylanase	*xlnA*	*A. nidulans*	*ACT*	-	2μ	*CYH2*	-	(Ganga *et al.*, 1999)
	Rhamnosidase	*rhaA*	*A. aculeatus*	*GPD*	*PGK*		*TRP*	-	(Manzanares *et al.*, 2003)
Increase volatile phenol aromas	Phenolic acid decarboxylase	*pdc* *padc*	*L. plantarum* *B. subtilis*	*PGK1*	*PGK1*		*URA3*	+	(Smit *et al.*, 2003)

Increase aroma-enhancing thiols from cysteinylated precursors	Tryptophanase with cysteinelyase activity	*tnaA*	*E. coli*	*PGK1*	*PGK1*		*SMR1-410*	+	(Swiegers *et al.*, 2007)
Modulate acetate ester aromas	Carnitine acetyltransferase	*CAT2*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*URA3*	-	(Cordente *et al.*, 2007)
	Alcohol acetyltransferase	*ATF1, ATF2*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*SMR1-410*	-	(Lilly *et al.*, 2000; Lilly *et al.*, 2006a)
	Ethanol hexanoyl transferase	*ETH1*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*SMR1-410*	-	(Lilly *et al.*, 2006a)
	Esterase	*IAH1*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*SMR1-410*	-	(Lilly *et al.*, 2006a)
Increase aromas associated with higher alcohols	Amino acid transaminase	*BAT1, BAT2*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*SMR1-410*	-	(Lilly *et al.*, 2006b)
Increase glycerol formation	Glycerol-3-phosphate dehydrogenase	*GPD1*	*S. cerevisiae*	*ADH1*	*ADH1*	2μ	Tn5 *ble*	-	(Michnick *et al.*, 1997; Remize *et al.*, 1999)
Reduce malic acid concentration	Malolactic enzyme	*mleS*	*L. lactis*	*PGK1*	*PGK1*	2μ	*URA3*	-	(Volschenk *et al.*, 1997b)
	Malate permease	*mae1*	*S. pombe*				*SMR1-410*	+	(Volschenk *et al.*, 2001)
	Malic enzyme	*mae2*							
	Malate permease	*mae1*	*S. pombe*	*PGK1*	*PGK1*		*URA3*	+	(Husnik *et al.*, 2006)
	Malolactic enzyme	*mleA*	*O. oeni*	*PGK1*	*PGK1*		*URA3*	+	
Reduce acetic acid concentration	Acetaldehyde dehydrogenase	*ALD6* deletion	*S. cerevisiae*				*kan MX4*		(Remize *et al.*, 2000a)
Increase wine acidity by lactic acid production	Lactate dehydrogenase	*LDH*	*L. casei*	*ADH1*	*ADH1*	2μ	*G418*	-	(Dequin *et al.*, 1999)
Decrease hydrogen sulphide synthesis	Sulphite reductase	*MET10*	*S. cerevisiae*	Site – directed mutagenesis (lowering enzymatic activity)					(Sutherland *et al.*, 2003)
Increase production of the antioxidant resveratrol	ß-glucosidase	*bglN*	*C. molischiana*	*ACT*	*ACT*	2μ	*CYH2*	-	(Gonzalez-Candelas *et al.*, 2000)
	Resveratrol synthase	*4CL216*	*Hybrid poplar*	*ADH2*	*ADH2*	2μ	*URA3*	-	(Becker *et al.*, 2003)
	Coenzyme-A ligase	*vst1*	*Grapevine*	*ENO2*	*ENO2*	2μ	*LEU2*	-	
Reduce ethyl carbamate content	Urea amidolyase	*DUR1,2*	*S. cerevisiae*	*PGK1*	*PGK1*		*URA3*	+	(Coulon *et al.*, 2006)
Synthesis of antimicrobial enzymes or peptides	Pediocin	*PED1*	*P. acidilactici*	*ADH1*	*ADH1*	2μ	*URA3*	-	(Schoeman *et al.*, 1999)
	Leucocin	*LCA1*	*L. carnosum*	*ADH1*	*ADH1*	2μ	*URA3*	-	(du Toit and Pretorius, 2000)
	Chitinase	*CTS1*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*URA3*	-	(Carstens *et al.* 2003)
	Glucose oxidase	*GOX*	*A. niger*	*PGH1*	*PGK1*		*URA3*	+	(Malherbe *et al.*2003)

Increase degradation of filter-clogging polysaccharides	Endopoly-galacturonase	*PGU1*	*S. cerevisiae*	*PGK1*	*PGK1*		*LEU2*	-	(Vilanova *et al.*2000)
	Pectatelyase	*pelA*	*F. solani*	*ACT*	-	2μ	*CYH*	-	(Gonzalez-Candelas *et al.*, 1995)
	Xylanase	*XYN2*	*T. reesei*	*ADH2, SSA1*	*ADH2*		*SMR*	+	(Louw *et al.*, 2006)
	Glucanase	*END1*	*B. fibris-olvens*	*ADH1*	*TRP5*		*SMR*	+	
	Pectate lyase	*PEL5*	*E. chrys-anthemi*	*ADH1*	*TRP5*		*SMR*	+	
	Polygalacturonase	*PEH1*	*E. caroto-vora*	*ADH1*	*TRP5*		*SMR*	+	

P: promoter; T: terminator; Pla: Plasmid; M: Marker; Chr: Chromosomal integration.

In most cases strong promoters and terminators were used, derived from glycolytic enzymes that are constitutively expressed under fermentative conditions (*ADH1*, *ADH2* and *PGK*), but also from the actin gene (*ACT*). A promoter collection comprising 11 mutants of the strong constitutive *S. cerevisiae TEF1* promoter has been recently constructed, that were used for fine-tuning of gene expression across a full continuum of possible expression levels. The activities of the mutant promoters range between about 8% and 120% of the activity of the unmutated *TEF1* promoter. In addition, promoter replacement cassettes were constructed that enable genomic integration of the mutant promoter collection upstream of any given yeast gene, allowing detailed genotype-phenotype characterizations (Nevoigt *et al.*, 2006).

Industrial yeasts usually do not have auxotrophic markers (e.g. *LEU2*, *URA2*), therefore the yeast-derived cycloheximide resistance gene *CYH2* or heterologous drug-resistance markers were used such as *ble* (from bacterial transposon Tn5, coding for a bleomycin binding protein) or *G418* (from bacterial transposon Tn903, coding for aminoglycoside phosphotransferase), conferring resistance to phleomycine and geneticine, respectively.

Plasmid-encoded genes should be preferably integrated, since the inserted elements have to be stable in the newly constructed organism, but such approaches were used in few cases (Volschenk *et al.*, 2001; Malherbe *et al.*, 2003; Smit *et al.*, 2003; Coulon *et al.*, 2006; Husnik *et al.*, 2006; Louw *et al.*, 2006). One-step gene disruption with auxotrophic markers as performed for the *GPD* gene (Michnick *et al.*, 1997) results in a self-cloning strain, a much less problematic approach in terms of acceptability evaluation according to the guidelines of the International Life Science Institute Europe (ILSI, 1999).

For heterologous expression of extracellular proteins, for example the *pedA*-encoding pediocin or *gox*-encoding glucose oxidase, secretion was usually directed by the mating pheromone α factor's secretion signal ($MFa1_S$) (Schoeman *et al.*, 1999; Malherbe *et al.*, 2003; Louw *et al.*, 2006).

The introduced modifications should not change essential characteristics of the host in the fermentation process. For most genetic modifications, it could be shown that apart from the introduced metabolic change, no significant differences were found between wines produced with commercial strain and the corresponding modified strain regarding their oenological characteristics. However, it was also shown that metabolic engineering can be associated with carbon flux re-routing and accumulation of undesirable byproducts such as pyruvate, acetate, acetoin and 2,3-butanediol (Michnick *et al.*, 1997), glycerol, succinate and butanediol (Remize *et al.*, 2000a) acetoin (Cambon *et al.*, 2006) or acetaldehyde, acetate and acetoin (Heux *et al.*, 2006b).

Other strategies, for example site-directed mutagenesis of the sulfite-reductase *MET10* gene were used to develop wine yeast with lowered ability to produce hydrogen sufide (Sutherland *et al.*, 2003). The allele *LEU4-1* confers resistance to 5,5,5-trifluoro-DL-leucine and the corresponding strains produce twice the amount of isoamyl-alcohol in laboratory-scale fermentations as the respective parental strains (Bendoni *et al.*, 1999).

Very recently, self-cloning gene manipulations, in which genes of a microorganism are cloned within the microorganism itself, overcame the topic of the commercial application of genetically modified yeasts. A self-cloning GMY sake strain (Akada, 2002), was approved by the Japanese Government, where a two-step gene replacement was used for the construction of a strain free of bacterial and drug-resistant marker sequences. A point mutation (Gly1250Ser) in the yeast fatty acid synthetase *FAS2* confers cerulenin resistance and is associated with a higher production of the apple-like flavor component ethyl caproate in Japanese sake. A novel counter-selection marker was used, that consisted of a galactose-inducible overexpression promoter and the *GIN11* growth inhibitory sequence (*GALp-GIN11*). Cells that retain the marker do not grow on galactose because of the growth inhibitory effect mediated by *GIN11* overexpression. A plasmid containing the mutated *FAS2* gene, a drug resistance marker and the counter-selectable marker was integrated into the wild-type *FAS2* locus, and the loss of plasmid sequences from the integrants was done by growth on galactose, which is permissive for the loss of *GAL*p-*GIN11*. Counter-selected strains contained either the wild type or the mutated *FAS2* allele, but not the plasmid sequences, and the resulting difference between the described mutant and the corresponding wild type strain is a single base (Akada *et al.*, 1999; Aritomi *et al.*, 2004).

The potential of whole cell engineering approaches

Important oenological traits, such as fermentative vigour, ethanol yield and tolerance, and growth temperature profile are not the result of single genes, but rather of a multitude of loci (quantitative trait loci, QTL), that are not well characterized because they are broadly distributed throughout the genome, and are involved in specific metabolic pathways or complex genetic networks. As mentioned in previous sections, the "classical" strain development approach

relies on repeated cycles of genetic diversity creation through mutagenesis and/or genetic recombination followed by selection or screening of desired phenotypes. This methodology has a long-standing history of successful application and increased acceptability from regulatory perspective compared to newer recombinant DNA based technologies. By blending classical methods of strain isolation with more recent approaches of cell engineering, coupled to high throughput screening and analytical technologies such as robotic miniaturization of assays and liquid chromatography mass spectrometry, the achievement of multiple complex, polygenic phenotypes of industrial yeasts will be speeded up in the in the post-"omics" era (Patnaik, 2007).

Cellular systems have the capacity to self-regulate their thousands of genes through fine-tuning of components of global transcription machinery. Global Transcription Machinery Engineering (gTME) uses a mutant transcriptional factor that perturbs the whole transcriptome in subtle ways, resulting in altered expression of polygenic phenotypes that are typically distributed on loci throughout the entire genome and thus provides a route to complex phenotypes that are not readily accessible by traditional methods (Alper and Stephanopoulos, 2007; Tyo *et al.*, 2007). This very promising advance of engineering one or few regulatory genes that belong to downstream branches of regulatory pathways could lead to subtle balancing of pathway networks without significant genetic intervention at the local enzyme or pathway level. Mutagenesis of the transcription factor Spt15p has been used for engineering of ethanol tolerance in yeast by reprogramming gene transcription to elicit more efficient glucose conversion and increased ethanol tolerance (Alper *et al.*, 2006).

Genome shuffling, where more than two parental strains per generation are enrolled, considerably improves the efficiency of protoplast fusion, which addresses only two parents per generation. The amount of genetic information used as starting point for the selection program is considerable enlarged, depending on the number of strains used for protoplast fusion. Recursive genomic multiparental recombination by protoplast fusion within a genetically heterogeneous population efficiently generates combinatorial libraries for selection or screening of improved, non-recombinant strains, in contrast to classical breeding approaches that allow for two parents mating (Patnaik *et al.*, 2002; Zhang *et al.*, 2002). Once a beneficial mutation is accumulated in a host background, recombination due to protoplast fusion between multiple parents evaluate the synergistic effect of that mutation in the background of all other beneficial traits already present in the population, without the need to generate all possible combinations experimentally. Multiparental genome shuffling has mainly been attempted with bacteria, such as improving titers of synthesized products that use complex pathways in *Streptomyces*, *Lactobacillus*, *Sphingobium*, and *E. coli*. In all these cases, genetic diversity was generated using classical mutagenesis techniques such as NTG, UV, and/or chemostat enrichments, followed by recursive protoplast fusion of mutant populations and screening or selection of the desired phenotypes (Patnaik, 2007). In yeast,

genome shuffling has been used to improve acetic acid tolerance of ethanologenic *Candida krusei*. The mutant isolated after four rounds of genome shuffling showed not only improved acetic acid, but also higher ethanol production and a superior multiple stress tolerance to ethanol, H_2O_2, heat, and repeated freeze-thaw cycles. This approach seems very promising for the rapid evolution of complex phenotypes in wine yeasts, such as fermentative vigor or ethanol yield and tolerance, which depend on a multitude of not well characterized QTL loci (Giudici *et al.*, 2005). However, a bottleneck in the application of this technique is the availability of high-throughput screening methods for the identification of complex phenotypes from a huge library of functional recombinant strains. Since genome shuffling is based on the principles of natural recombination, combined with an accelerated evolutionary approach, the resulting organisms cannot be considered as genetically modified.

Adaptive evolution is a non-recombinant means of strain improvement that consists in culturing a population for many generations under conditions to which it is not optimally adapted. The yeast genome adapts with flexibility to the externally introduced environmental changes. This has been demonstrated by culturing a clonal population of a diploid strain under glucose-limited conditions for 250 generations in three separate experiments. The evolved strains were fitter genetic variants in comparison to the founding strain, with significantly altered gene expression that affected several hundred genes, as was shown by transcriptome analysis. Many genes were identical in the three replicates, causing comparable improvements in glucose utilization, most probable through point mutations in a few key regulatory genes rather than gross genomic rearrangements (Ferea *et al.*, 1999). Chemostat enrichment was used to select for gain-of-function mutants with mutations in the biodesulfurization (Dsz) system of *Rhodococcus erythropolis* IGTS8, enriching for growth in the presence of organosulfur compounds that could not support growth of the wild-type strain (Arensdorf *et al.*, 2002). By evolutionary adaptation of the commercial wine yeast L-2056 in sequential batch fermentations during 200 – 500 generations in a wine-like medium, strains were obtained that catabolized more rapidly all available sugars and also showed an altered production of metabolites, such as ethanol, glycerol, succinic and acetic acid. (McBryde *et al.*, 2006). Similar approaches were used to obtain multiple-stress (oxidative, freezing–thawing, high-temperature and ethanol resistance) phenotypes in *S. cerevisiae* (Cakar *et al.*, 2005). Recent theoretical work suggests that most of the phenotypic change during an episode of adaptation can result from the selection of a few mutations with relatively large effects (Zeyl, 2005). Evolutionary engineering uses a combination of heterologous expression or targeted knock-outs followed by directed evolution under growth-selective conditions. Evolutionary engineering was successfully used to obtain genetically enhanced xylose degrading *S. cerevisiae* strains, by first assembling a heterologous xylose pathway followed by prolonged evolution under selective conditions (Sonderegger and Sauer, 2003; Kuyper *et al.*, 2004; Kuyper *et al.*, 2005; Jeffries, 2006).

Genomic and proteomic studies of *S. cerevisiae* under winemaking conditions and their potential for obtaining novel GMY

Recent advances in high-throughput experimental techniques supported by bioinformatics have resulted in rapid accumulation of a wide range of transcriptomics and proteomics data that provide a foundation for in-depth understanding of how cells respond to changing environments. DNA microarray technology (''DNA chips'') (Schena *et al.*, 1995; DeRisi *et al.*, 1996) has found widespread use as most flexible tool to investigate genome-wide comprehensive data on the transcriptional response of the whole yeast genome in different metabolic states and revealed networks of coordinated regulation. Wine fermentation is clearly one example of a process in which yeast cells have to adapt to stressful initial conditions, and need to cope with significant nutritional variations throughout the whole process.

When dry active yeast cells are inoculated into the must, they need to deal with hyperosmotic stress due to the high sugar concentration in this medium (*ca.* 160 – 250 gl^{-1}, as equimolar mixture of glucose and fructose). Fermentation progression creates ethanol and nutrient limitation, while the cell is subjected to several potential stress factors such as temperature shifts, high CO_2 and SO_2 levels, and the presence of competing organisms. Commercial yeast strains used in winemaking must be able to maintain fermentative capability and cellular viability under these multiple stressful conditions. Since many physiological traits are consequences of complicated multigene regulation, understanding the way they are expressed, not only during wine fermentation, will contribute to the knowledge about the genetic make-up of commercial yeast strains and will also influence wine strain improvement by genetic engineering. Comparative genomics will elucidate cellular processes that are associated to fermentation arrest and other technological problems caused by the yeast metabolism and will also be useful to show that the genetic modifications are not associated with adverse or unexpected side-effects such as the production of toxic substances.

Global gene expression after a short-term ethanol stress (30 minutes) was associated with up-regulation of 3.1% and down-regulation of 3.2% of the yeast genes (factor 3 in both cases). Cellular adaptation mechanisms involved, besides the stress gene family, energy metabolism regulation, ionic homeostasis, heat protection, trehalose synthesis and antioxidant defense (Alexandre *et al.*, 2001). Transcriptome analysis was also completed with the widely commercialized oenological *S. cerevisiae* strain EC1118 from Lallemand S.A., at different time points during alcoholic fermentation using a synthetic must medium. Transcriptional reprogramming was characterized by a tightly controlled and coordinated regulation, affecting more than 2000 genes, in consequence of cellular adaptation to changing nutritional, environmental and physiological conditions. Initial stresses such as high osmotic pressure and acidity did not trigger stress response, which is surprising, given the large amounts of sugars in the fermentation medium and the corresponding high osmotic pressure. Superimposition of multiple stresses (ethanol, osmotic, acid, nutrient depletion)

during entry into the stationary phase was associated with a unique stress response (Rossignol *et al.*, 2003). There is a need to deepen molecular and biochemical aspects of the rehydration process in order to better understand the factors contributing to a quick fermentation by the inoculated strain. A detailed analysis was performed to study the transcriptome of wine yeast before and after rehydration and during the first hours following inoculation of a synthetic must. The transcriptome of commercialized dry yeast cells corresponded to a physiological stage of respiration, nitrogen and carbon source starvation, and high stress. During rehydration, a process that usually occurs at 35-40°C during 30 min in a concentrated sugar solution, yeast quickly recovered the capacity to respond appropriately by coordinate induction of genes involved in biosynthetic pathways (e.g. nitrogen utilization), transcription or protein synthesis, while genes associated with the general stress response or subject to glucose repression were down-regulated. Despite the high sugar concentration, no osmotic stress response was triggered at the initial stages (Rossignol *et al.*, 2006). Similar results were obtained in a study evaluating the early transcriptional responses of wine yeast after rehydration in water followed by transfer to synthetic must medium, a solution containing fermentable carbon sources, or a sorbitol solution corresponding to the osmotic pressure of the synthetic must medium (Novo *et al.*, 2007).

Global transcriptional profiling was also used to elucidate metabolic pathways in *S. cerevisiae* under conditions used for the production of dessert wines, where the initial sugar concentration may be as high as 50% (w/v). Cells cope with such very stressful fermentation conditions by up-regulating glycolytic and pentose phosphate pathway genes, being the latter proposed as a shunt for glucose-6-phosphate and fructose-6-phosphate, from the glycolytic pathway (Erasmus *et al.*, 2003).

Fermentation at lower temperatures is an important parameter that can contribute to improved aromatic complexity of wines, by increasing biosynthesis of flavor-active compounds such as volatile esters and medium-chain fatty acid esters, a greater retention of terpenoid compounds, a reduction in higher alcohols and volatile acidity (Lambrechts and Pretorius, 2000; Torija *et al.*, 2003). Comparative transcriptional changes were evaluated in the commercial strain QA23, conducting industrial fermentations at the "optimal" temperature of 25°C versus 13°C. Expression profiles during wine fermentation at 25°C contrasted significantly with those at 13°C, 535 ORFs were significantly differentially expressed. At 13°C, a characteristic cold-stress response was apparent, associated with genes responsible for increased production of short-chain fatty acids and their esters, where a strongly induced esterase/ester synthase encoded by *IAH1* might be involved. Low fermentation temperature was also linked to higher cell viability and improved ethanol resistance (Beltran *et al.*, 2006).

Grape musts are often unbalanced, with nitrogen sources, lipids and vitamins as limiting components. All these conditions lead to gene expression changes of yeast cells as they are challenged to adapt to these extreme nutritional

stress. Nitrogen metabolism provides precursors for protein biosynthesis, but also of compounds that determine the final wine's organoleptic properties (Lambrechts and Pretorius, 2000). Nitrogen limitation affects metabolic activities of growing or non-growing cultures and leads to problematic enological fermentations such as stuck or sluggish fermentation (Bisson, 1999). The typical oenological method to prevent problems due to nitrogen-limitation is the early addition of ammonium salts to the grape juice. This practice triggers the down-regulation of genes coding for small molecule transporters and nitrogen catabolic enzymes, including those linked to the production of urea, a precursor of ethyl carbamate in wine, which is a compound with genotoxic properties *in vivo* and *in vitro*. Up-regulated genes were involved in protein synthesis, amino acid metabolism, purine biosynthesis and sulfate assimilation (Marks *et al.*, 2003). Transcriptomic differences were also evaluated in typical fermentations with high compared to low nitrogen source (arginine). At the beginning of fermentation, usually in high nitrogen conditions, fermentative activity is high and the glycolytic enzymes show high expression levels, which corresponds to a typical fermentative process with anaerobic pathways acting. Contrarily, in low nitrogen condition, a partial alleviation of glucose repression, despite high (17% w/v) external glucose concentrations was observed, combined with enhanced expression of ribosomal protein genes and reduced expression of genes involved in carbohydrate and nitrogen metabolism. Under these conditions, a switch from fermentation to a functional oxidative glucose metabolism was observed, suggesting that respiration is more nitrogen-conserving than fermentation (Backhus *et al.*, 2001). When a nitrogen-deficient fermentation was nitrogen-supplemented (200 mg/l, supplied as diammonium phosphate), cells were able to overcome the previous nitrogen starvation stress and restarted alcoholic fermentation, with a concomitant upregulation of many genes involved in glycolysis, thiamine metabolism, and energy pathways (Mendes-Ferreira *et al.*, 2007b). From this work, 36 genes were identified, that were highly expressed under conditions of low or absent nitrogen in comparison with a nitrogen-replete condition, that can be used as signature genes for prediction of stuck or sluggish fermentations (Mendes-Ferreira *et al.*, 2007a).

Data generated from genome-wide research of DNA sequence variation, combined with studies that have examined variation at the transcriptional and physiological level under winemaking conditions, all suggest that populations of *S. cerevisiae* harbor large amounts of genetic variation. Zuzuarregui *et al.* (2005) determined expression patterns of some stress response genes induced in 24 efficient commercial wine yeast strains after 30 minutes of inoculation in a synthetic culture medium containing 20% (w/v) of glucose. Strain-specific high *GPD1* expression occurred, in agreement with the need of glycerol production for osmotic adjustment to counteract hyperosmotic stress present at the beginning of vinification. Contrarily, other stress genes (*TRX2*, *HSP104* and *SSA3*) were weakly induced. Augmented expression occurred with decreasing temperature or increasing pH. Comparison of strains with different fermentative behavior also indicated that very low expression levels for stress response genes can result in

viability problems, but very high levels cause growth defects (Zuzuarregui *et al.*, 2005). These kind of data are useful in choosing the most appropriate strain to be inoculated for wine production, depending on must pH and desired fermentation temperature. The commercial strains ICV 16 and ICV 27 share similar characteristics at initial fermentative stages, but only strain ICV 16 is able to complete fermentation in a synthetic must medium. A comparative integrated transcriptomic-proteomic analysis of both strains revealed associations between fermentative behaviors and differences in mRNA and protein profiles. In strain ICV 16, an increase in the abundance of proteins involved in carbohydrate metabolism (in particular, the major cytosolic aldehyde dehydrogenase Ald6p) and in stress responses (e.g. the heat shock protein Hsp26p) may explain the better behavior during vinification and also a better adaptation to the stationary-phase conditions, leading to a fully completed vinification. In strain ICV 27, higher levels of enzymes involved in the sulfur assimilation pathway (Cys4p, Hom6p, and Met22p) were found, that might be related to the production of particular organoleptic compounds, explaining the strain's commercial interest for the aromatic profile of wines (Zuzuarregui *et al.*, 2006). This work clearly shows the potential of both transcriptomic and proteomic approaches to elucidate the molecular bases of differential physiological response and traits that are important in winemaking.

Transcriptome analysis is a straightforward approach to identify candidate genes for genetic improvement, followed by their overexpression, as was recently shown for a Japanese rice wine (sake) strain under high NaCl concentration or ethanol stress (Hirasawa *et al.*, 2006, Hirasawa *et al.*, 2007). Improvement strategies of industrial properties of yeast used for fermentation will greatly benefit from functional genomics and the generated knowledge about the inter-related regulatory and metabolic processes. In this way, links between genes, pathways and phenotypes will become more evident and predictive models can be also generated. Furthermore, data integration will make it possible to identify and modify upstream regulators of metabolic pathways and regulatory networks as an alternative approach to the conventional approach of targeting of few genes that belong to downstream branches of regulatory pathways. The holistic systems biology views of the cell coupled with further development of "omic" technologies will greatly benefit winemaking and other yeast-based industrial processes.

The future of wines obtained from genetically modified yeast

Legal aspects

In May 1997, the European Regulation EC258/97 on novel foods and novel food ingredients (EC, 1997) came into force and includes within its scope foods and food ingredients containing or consisting of genetically modified organisms (GMO) or produced by GMO, whereas these are not present in the food. The safety of a food derived from a GMO has to be evaluated by comparing it with

the most similar food, which has a history of safe use. This means that, if a food derived from a GMO is substantially equivalent, it is "as safe as" the corresponding conventional food item and should be treated as such, whereas identified differences are the subject for further toxicological, analytical and nutritional investigations. Detailed knowledge of both the overall characteristics and genetic background of the organisms, the source of the transferred gene(s) and the function of the modified genes is essential for this evaluation. The final outcome of a genetic modification is based on processes that are controlled by numerous different genes, whereas the function of many genes is still poorly understood. Powerful methods for the identification and characterization of unintended effects on a genomic, proteomic and metabolomic scale are therefore evaluated for their routine use (Kuiper *et al.*, 2002; Kuiper and Kleter, 2003; Corpillo *et al.*, 2004).

The Novel Food Regulation has been recently amended by three new regulations concerning GMO, including derived foods and feeds: EC1829/2003 (EC, 2003a), 1830/2003 (EC, 2003b) and 65/2004 (EC, 2004), which define the procedures for authorization, labeling and traceability. Regulation 1829/2003 describes the information to be provided by an applicant seeking authorization to place a product on the market. The applicant has to show that the referred food must not (i) have adverse effects on human and animal health and the environment, (ii) mislead the consumer and (iii) differ from the food, which it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for the consumer. Such products must undergo a safety assessment before being placed on the market, including a technical dossier with detailed information concerning results obtained from research and developmental releases to evaluate the GMOs impact on human health and environment. This is defined in Annex III of Directive 2001/18/EC (EC, 2001) on the deliberate release into the environment of GMO for placing on the market or for any other purpose, that repealed the former Council Directive 90/220/EC (EC, 1990). Since placing on the´market includes deliberate release into the environment, an environmental risk assessment in accordance with Annex II of Directive 2001/18/EC has to be carried out (EC, 2002). The product then goes through the approval procedure between the European Food Safety Agency (EFSA) in Brussels, the European Commission and member states. Labeling is mandatory, even if the recombinant DNA or the corresponding protein cannot be detected in the final product. Foods containing GMOs have to be labeled "genetically modified" or "produced from genetically modified (name of the ingredient)". Labeling is not required for foods containing traces of GMOs, which are adventitious and technically unavoidable, in a proportion lower than the threshold of 0.9% of the food ingredients (relation between recombinant and non-recombinant ingredient). The Novel Food Regulation was based on the principle of evidence, in the sense of mandatory labeling for food products containing more than 1% GMOs, whereas Regulation EC1829/2003 is supported by the principle of application, making the declaration of GMO use during the production of food compulsory. According to Regulations N° 1830/2003 (EC,

2003b) and 65/2004 (EC, 2004), GMOs and products derived from GMOs must be traceable during all stages of their placing on the market through the production and distribution chain, to facilitate withdrawal of products when necessary and to facilitate the implementation of risk management measures. Mention should also be made to the regulation 65/2004 and which establishes a system for the creation and assignment of unique identifiers for GMO.

USA regulations do not require mandatory labeling and segregation of genetically modified products. No special labeling is required for "bioengineered foods", the term used by FDA for those derived by genetic modification (GM) technology, "as they are not considered to differ from other foods in any meaningful or uniform way or, as a class, to present any different or greater safety concern than foods developed by traditional plant breeding" (Federal Register of May 29, 1992 57 FR 22984). Evaluation and approval before marketing is only required when the introduced gene encodes a product that had never been a component of any other food, such as a new sweetening agent for example. The labeling requirements that apply to foods in general are therefore also relevant to foods using biotechnology. A label must "reveal all material facts" about a food, for example if a bioengineered food is significantly different from its traditional counterpart, has a significantly different nutritional property or if a potential allergen is present.

Wines produced by GMY should be, in general, considered as substantially equivalent to "traditional" wines. Compounds like glycerol, acetate ester, malic or lactic acid are natural wine substances, and their content would be merely adjusted or optimized in the sense of enhanced organoleptical characteristics. The expected concentration is very likely to lie within the range that can be found in different wine styles. New profiling methods using transcriptomics, proteomics and metabolomics were proposed as the most adequate non-targeted approaches to detect secondary effects (Kuiper and Kleter, 2003). Proteome analysis demonstrated "substantial equivalence" for the modified S. *cerevisiae* 522^{EC} strain, with constitutive expression of genes involved in urea degradation, that ultimately leads to decreased levels of ethyl carbamate, a potential carcinogenic agent for humans that is formed through the reaction of urea and ethanol (Coulon *et al.*, 2006). The malolactic yeast strain ML01, which contains the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *Oenococcus oeni* malolactic gene (*mleA*) under control of the *S. cerevisiae PGK1* promoter and terminator sequences is the first commercialized genetically enhanced *S. cerevisiae* wine yeast that has obtained approval by the FDA in the USA. Substantial equivalence has been demonstrated through phenotypic, genotypic and transcriptomic approaches (Husnik *et al.*, 2006).

Environmental risk assessment

According to the previously mentioned legal aspects, the future of GMY for wine production will also depend on the ability to assess potential risks associated with their introduction into natural ecosystems.

An experimental model has been used to assess the fate of GMY strains in natural environments by tracking the spreading of industrial yeast strains in vineyards close to the wineries where these strains were used during the last 5-10 years. These large-scale studies, carried out over a 3-years period in vineyards located in North Portugal and South France, revealed that dissemination of commercial yeast in the vineyard is limited to short distances and periods of times and is largely favored by the presence of water runoff. In samples taken at distances from wineries higher than 100 m, less than 2% of the fermentative microflora had a genetic profile identical to that of commercial yeast. In samples taken close to the winery and to water rills, the proportion of commercial yeasts increased to 10-43%. The vast majority (94%) of commercial yeasts were found at a distance of between 10 and 200 m from the winery (Valero *et al.*, 2005). Commercial strains, despite their intensive annual utilization, do not seem to implant in vineyards, and do not predominate over the indigenous flora, being their presence characterized by natural fluctuations of periodical appearance/ disappearance as has been described for autochthonous strains (Schuller *et al.*, 2005).

As summarized in Table 1.2, the genetic modifications in recently developed GMY strains by recombinant technologies refer mainly to altered gene dosage and introduction of new metabolic pathways and result in improved performance during fermentation. From our current knowledge, it does not seem likely that such strains would exhibit superior predominance and survival in nature, considering also that natural environments are very different from fermentative settings in terms of nutrient availability. The behavior of GMY strains within microbial populations of a confined wine cellar and greenhouse vineyard has been evaluated, to our knowledge, in only one study. From the commercial strain VIN13 different GMY strains were constructed, containing heterologous genes expressing α-amylase (*LKA1*), endo-β-1,4-glucanase (*end1*), xylanase (*XYN4*) or pectate lyase (*peh1*) under the control of strong promoters and terminators and using the *kanMX* or *SMR*-410 resistance markers. After initial characterization of the autochthonous yeast flora of the newly established greenhouse vineyard, the vines of four blocks (each consisting of 20 vines) were sprayed with yeast suspensions containing 2.5 x 10^6 CFU/ml according to a previously defined scheme. Despite the high initial cellular concentrations, only few *S. cerevisiae* strains were isolated during the weekly monitoring of yeast populations on grapes, leaves, stems and soil. Results showed that (i) no significant difference between the occurrence of the modified strains compared to the parental commercial strains was evident, even for genetically modified strains that were supposed to have a selective advantage over the parental strains (secreting glucanases and pectinases), showing that the above mentioned modifications did not confer any fitness advantage, (ii) the overall yeast populations on the sprayed blocks were very similar to the untreated control vines, leading to the conclusion that neither commercial strains nor GMY affect the ecological balance of vineyard-associated flora in a confined system, (iii) no significant differences among the strains were detected concerning their

fermentation performance during spontaneous micro-vinifications (Bauer *et al.*, 2003).

Consumer's perceptions, attitudes and concerns

Wine can be considered the alcoholic beverage where the image associated with "tradition" is a mostly important attribute for successful marketing, and gene technology may constitute a threat for the image of the product.

In 1988, Gist-Brocade obtained a baker's strain where the genes coding for maltose permease and maltase were substituted with a more efficient set of genes from another strain. Since no non-*Saccharomyces* DNA was present, the UK authorities granted consent in 1989. A few years later, a recombinant brewer's strain, obtained in 1993 by Brewing Research International was equally approved. This *S. cerevisiae* strain contained an amylase gene from *Saccharomyces diastaticus* together with a gene for copper resistance. Because of the unwillingness of the industries to face a negative consumer reaction, none of the strains has gone into commercial production (Moseley, 1999). For the same reasons, no application for the industrial use of genetically modified wine strains has been submitted in the last few years, although many strains were developed, as previously shown in Table 1.2.

One of the most extensive (in terms of the number of people surveyed) public opinion analysis in Europe is the Eurobarometer survey, that has been monitoring changes in attitude toward biotechnology in different European member states since the early 1990s. The last survey was conducted in 2005 (Gaskell *et al.*, 2006) and was based on a representative sample of 25,000 respondents, approximately 1,000 in each EU Member State. Issues such as stem cell research, the co-existence of GM, conventional and organic farming, the use of genetic information, and other innovations such as nanotechnology and pharmacogenetics were under discussion. In comparison to earlier surveys, the portrait of European citizens painted by the 2005 survey shows them with more optimistic, informed and trusting perceptions toward the previously mentioned range of biotechnologies. In contrast to previous surveys, there is no evidence that opposition to genetically modified food is a manifestation of a wider disenchantment with science and technology in general. However, a majority of Europeans sees GM food as not being useful, morally unacceptable, as a risk for society and therefore should not be encouraged (Gaskell *et al.*, 2006). The fears by the critics of GM technology include alterations in nutritional quality of foods, potential toxicity, possible antibiotic resistance, potential allergenicity and carcinogenicity from consuming genetically modified foods, environmental pollution, unintentional gene transfer, possible creation of new viruses and toxins, religious, cultural and ethical concerns, and fear from the unknown (Uzogara, 2000). There is no clear answer to the question whether there is a market for wines produced with GMY. A recent survey carried out in Slovenia regarding genetically modified organisms in winemaking showed that 65% of 334 interviewees disagree with the usage of genetically modified organisms in

winemaking. Disapproval by retailers was similar (64%) and by enologists lower (55%) (Plahuta *et al.*, 2007).

Summarizing, the recent availability of clear legal regulations defining requirements for construction and safety evaluation of genetically modified organisms and the labeling of products obtained by their use can be considered a crucial step to assist the consumer in making an informed choice, but the results from the latest Eurobarometer survey show that this was not sufficient to increase the consumers trust in genetically modified food. Within this scenario, we can estimate that genetically enhanced wine yeast strains obtained by strategies based on self-cloning or genome-scale approaches such as adaptive evolution, mimicking the principles of natural whole genome evolution in a laboratory setting, will fast-track strain improvement programs within the next future. In parallel, information obtained from the analysis of the genomes, transcriptomes, proteomes and metabolomes of a higher number of *S. cerevisiae* wine strains and data integration within systems biology approaches will reveal genetic characteristics that are responsible for the better suitability of certain strains to particular types and styles of wine.

Future perspectives

The accumulated knowledge of the *S. cerevisiae* cellular biology, biochemistry, physiology and genetics in combination with the amount of data generated from genome-wide research of DNA sequence variation and with studies that have examined variation at the transcriptional and physiological level under winemaking conditions, all suggest that populations of *S. cerevisiae* harbor large amounts of genetic variation. Using *S. cerevisiae* for millennia in wine-making may have created unique and interesting oenological traits and the community of wine yeast researchers is looking forward to unravel functional implications of genetic variation in *S. cerevisiae* strains used in winemaking, to reveal genetic features responsible for variation in fermentation properties and differing sensory characteristics in the final wine. It is particularly important to characterize the molecular mechanisms (or genetic constitution) that confers outstanding fermentation performance or multiple stress resistance to specific winemaking strains. This will permit the finding of links between selected winemaking strains and their contribution to specific sensory properties in finished wine, to investigate why certain strains are more suitable for specific wine styles and to search for common genetic features among *S. cerevisiae* strains used for winemaking and reveal the genetic characteristics that distinguish them from strains used for other biotechnological applications. It can be estimated that within the next few years the costs for genome-wide approaches will decrease and at the same time the number of sequenced *S. cerevisiae* strains will increase. A large field of investigation lies ahead, that will answer questions that are interesting from both fundamental and applied points of view.

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CHAPTER - 2

GENETICS AND GENOMICS OF *ASPERGILLUS FLAVUS*

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Introduction

Aspergillus flavus is the most infamous species among the over 185 known species within the genus *Aspergillus*. It is not only one of the most abundant and widely distributed soil-borne molds that can be found anywhere on earth but also produces aflatoxins, among the most carcinogenic natural products ever discovered (Jelinek *et al.*, 1989). *A. flavus* is a saprobe capable of surviving on many organic nutrient sources like plant debris, tree leaves, decaying wood, animal fodder, cotton, compost piles, dead insects and animal carcasses, stored grains, and even human and animal patients (Klich, 1998). Its optimal range for growth is between 25 - 37°C, but it can grow in a wide range of temperatures from 12 to 48°C. The ability of the fungus to grow at relatively high temperatures of the fungus contributes to its pathogenicity in humans and other warm blooded animals. For most of its life-cycle, the fungus exists in the form of

mycelia or asexual spores known as conidia. Stress from adverse conditions such as lack of adequate nutrients or water, causes the mycelia to form resistant structures called sclerotia. The fungus over-winters either as spores, as sclerotia or as mycelia in debris. Under favorable conditions sclerotia germinate directly to produce new colonies or conidiophores with conidia (Bennett *et al.*, 1986; Cotty, 1988; Chang *et al.*, 2002)

After *A. fumigatus*, *A. flavus* is the second leading cause of invasive and non-invasive aspergillosis in humans and animals (Denning *et al.*, 1991; Denning, 1998; Mori *et al.*, 1998; Denning *et al.*, 2003). The incidence of aspergillosis is rising due to the increase of immunocompromised patients in the population (Denning, 1998; Nierman *et al.*, 2005; Ronning *et al.*, 2005). Moreover, *A. flavus* is a weak and opportunistic plant pathogen, affecting many agricultural crops such as maize (corn), cotton, groundnuts (peanuts), as well as tree-nuts such as Brazil nuts, pecans, pistachio nuts, almonds and walnuts. It can contaminate these crops with the secondary metabolite aflatoxins in the field before harvest. Aflatoxin has been named after *Aspergillus flavus* toxin. It also causes the spoilage of post harvest grains during storage (St Leger *et al.*, 2000). Under weather conditions favorable for its growth, *A. flavus* can cause ear rot on maize, resulting in significant economic losses to farmers (Robens, 2001; Robens and Cardwell, 2005).

Aspergillus parasiticus, a sibling species to *A. flavus*, produces aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFG_1, AFB_2, and AFG_2). These four major aflatoxins are named based on their blue (B) or green (G) fluorescence under ultraviolet light, and their relative mobility by thin-layer chromatography on silica gel. Aflatoxin M_1 is a hydroxylated derivative metabolized from aflatoxin B_1 by cows and secreted in milk (Van Egmond, 1989). *A. flavus* produces aflatoxins B_1 and B_2. In addition, it also produces many other mycotoxins such as cyclopiazonic acid, kojic acid, beta-nitropropionic acid, aspertoxin, aflatrem and aspergillic acid (Goto *et al.*, 1996).

Of the aflatoxin family of metabolites, aflatoxin B_1 is the most toxic as well as the most potent carcinogen. Aflatoxin was identified as a food poison after a mysterious "Turkey-X" disease killed 100,000 turkey poults in hatcheries in England in 1960 after being fed with *A. flavus* infested peanut-meal (Allcroft *et al.*, 1961; Lancaster *et al.*, 1961). The disease caused by ingestion of aflatoxins in contaminated food or feed is called aflatoxicosis. Acute aflatoxicosis may occur when moderate to high levels of aflatoxins are consumed causing acute liver damage, acute necrosis, cirrhosis, or in severe cases, acute liver failure and death (Fung and Clark, 2004; Lewis *et al.*, 2005).

Major outbreaks of acute aflatoxicosis from contaminated food in humans have been documented in developing countries (CDC, 2004; Lewis *et al.*, 2005). For example, in western India in 1974, 108 persons died among 397 people affected with aflatoxin poisoning in more than 150 villages (Krishnamachari *et al.*, 1975). A more recent incident of aflatoxin poisoning occurred in Kenya in July 2004 leading to the death of 125 people among 317 reported illness due to

consumption of aflatoxin contaminated maize (corn) (Krishnamachari *et al.*, 1975; CDC, 2004; Lewis *et al.*, 2005). Acute toxicosis is not the only concern. World health authorities warn that low doses with long term dietary exposure to aflatoxins is also a major risk as this can lead to hepatocellular carcinoma (Bressac *et al.*, 1991; Hsu *et al.*, 1991; Wogan, 1992; Fung and Clark, 2004).

In summary, aflatoxin contamination of agricultural commodities poses a potential risk to livestock and human health (Lancaster *et al.*, 1961; Bennett and Lee, 1979; Bennett, 1987; Jelinek *et al.*, 1989; Cleveland and Bhatnagar, 1992; Eaton and Groopman, 1994; Hall and Wild, 1994; Bhatnagar *et al.*, 2002; Bennett and Klich, 2003; Yu *et al.*, 2007a). It is not only a serious food safety concern, but it also has significant economic implications for the agricultural industry worldwide because of restrictions limiting the trade of contaminated crops. These concerns have lead to extensive studies on the occurrence, biosynthesis, and toxicity of aflatoxins (Van Egmond, 1989; Yu *et al.*, 2004a; Yu *et al.*, 2004b; Yu *et al.*, 2004c; Yu, 2004; Yu *et al.*, 2006). Over the last two decades a major focus has been on understanding the genetic basis of aflatoxin biosynthesis. These studies began with mutagenesis and classical genetic analyses which led to development of a series of strains having mutations in the aflatoxin biosynthetic pathway (Papa, 1976; Papa, 1979; Papa, 1984). Development of new technologies including those of molecular biology allowed researchers to clone the specific genes that are directly involved in aflatoxin biosynthesis (Chang *et al.*, 1992; Skory *et al.*, 1992; Chang *et al.*, 1993; Yu *et al.*, 1993; Yu *et al.*, 1995; Chang *et al.*, 1995a; Chang *et al.*, 1995c; Cary *et al.*, 1996; Yu *et al.*, 1998; Chang and Yu, 2002; Yu *et al.*, 2004c; Yu *et al.*, 2004a). More recently, technological breakthroughs in large scale DNA sequencing has made it possible to study whole *Aspergillus* genomes (Yu *et al.*, 2004d; Yu *et al.*, 2005; Machida *et al.*, 2005; Kim *et al.*, 2005; Yu *et al.*, 2006; Payne *et al.*, 2006; Price *et al.*, 2006; Yu *et al.*, 2007a). Significant progress has been made in deciphering the aflatoxin biosynthetic pathway, genes involved in aflatoxin biosynthesis, and the *A. flavus* genome structure (Payne and Brown, 1998; Yu *et al.*, 2004c; Yu *et al.*, 2005; Payne *et al.*, 2006). In this chapter, we summarize the current progress in understanding the genetics and genomics of *A. flavus*.

Genetics of *Aspergillus flavus*

Genetic linkage groups

Genetic studies on *A. flavus* and *A. parasiticus* have been hindered by the fact that these fungi do not have a known sexual stage. However, they are able to undergo genetic recombination parasexually (Papa, 1973; Gussack *et al.*, 1977; Papa, 1978; Bennett *et al.*, 1979). The property of parasexual recombination was used in early genetic studies of the aflatoxin-producing species *A. flavus* and *A. parasiticus* (Papa, 1973; Papa, 1976; Papa, 1978; Papa, 1979; Papa, 1984).

In the early 1970s, numerous aflatoxin nonproducing mutants of *A. flavus* (*afl*) were generated by chemical agents such as N-methyl-N'-nitro-N-nitrosoguanidine. Over 36 genes were mapped to eight (8) linkage groups (LGs). The identified aflatoxin (*afl*) mutants of *A. flavus* were mapped to linkage group VII. These included *afl-1*, *afl-15*, *afl-16*, *afl-17*, *afl-19*, *afl-20*/*afl-22*, *afl-21*, *afl-25*, *arg-7* (arginine), *leu-7* (leucine), *aflR* (*afl-2*), and *nor-1* genes (Papa, 1984). The mutant *afl-4* was mapped to LG II and *aflB2* mapped to LG VIII (Papa, 1977; Papa, 1979). All of these mutations were shown to be recessive except for one dominant allele, the *afl-1* (Papa, 1980). Later, this dominant mutant was demonstrated to contain a deletion of about 120 kb, within which the 75 kb aflatoxin pathway gene cluster resides (Woloshuk *et al.*, 1995; Yu *et al.*, 2004c; Smith *et al.*, 2007). Recent studies revealed that *A. parasiticus* is capable of producing sexual spores through sexual recombination (Horn *et al.*, 2009). Crosses between strains with opposite mating-type genes MAT1-1 and MAT1-2 resulted in the development of ascospore-bearing ascocarps embedded within stromata. A sexual cycle was also discovered in *A. fumigatus* also believed earlier to be a asexual species (O'Gorman *et al.*, 2008). These discoveries are very important for fungal biology and evolution.

Chromosome karyotypes

The *A. flavus* and *A. parasiticus* chromosome structures have been studied by electrophoretic karyotyping (Keller *et al.*, 1992; Foutz *et al.*, 1995). Six LGs as defined by Bennett and Papa (Bennett and Papa, 1988) were assigned to five *A. flavus* chromosomes (Foutz *et al.*, 1995). The two markers genes, *arg7* and *leu7*, and the important aflatoxin pathway gene (*nor-1*) and the pathway regulatory gene (*aflR*) were mapped to a 4.9-Mb chromosome (Foutz *et al.*, 1995). Electrophoretic karyotyping indicated that both *A. flavus* and *A. parasiticus* contain at least 7 large chromosomes (Keller *et al.*, 1992; Foutz *et al.*, 1995) ranging from 3.0 to 7.0 Mb. The 7.0 Mb large chromosome was stained more intensely than the rest implying an unresolved double chromosomes of identical size. The genome size was estimated to be 36 Mb (Keller *et al.*, 1992; Foutz *et al.*, 1995) These results are consistent with the currently assembled genome sequence data, based on optical mapping, in that the *A. flavus* genome contains eight chromosomes and is about 36.8 Mb in size (Payne *et al.*, 2006; Yu *et al.*, 2007a).

Genetics of aflatoxin biosynthesis

Aflatoxin biosynthetic pathway genes

Intensive research efforts have focused on elucidation of the pathway of aflatoxin biosynthesis. This pathway is now one of the best-studied pathways of fungal secondary metabolism. The establishment of aflatoxin biosynthetic pathway was aided by the hallmark discovery of a color mutant that accumulates

a brick-red pigment in *A. parasiticus* (Lee *et al.*, 1971; Bennett *et al.*, 1971; Bennett *et al.*, 1976). The colored compound in this mutant, norsolorinic acid, turned out to be the earliest, stable aflatoxin precursor (Barnes *et al.*, 1994; Bennett *et al.*, 1997). The major biochemical pathway steps and aflatoxin pathway intermediates have been elucidated (Minto and Townsend, 1997; Payne and Brown, 1998; Yu *et al.*, 2004a; Yu, 2004; Yu *et al.*, 2004c; Yu *et al.*, 2005). At least 23 enzymatic reactions are estimated to be involved in aflatoxin formation. No less than 15 structurally-defined aflatoxin intermediates have been identified in the aflatoxin/sterigmatocystin (ST) biosynthetic pathway (Bennett and Klich, 2003; Yu *et al.*, 2004c; Yu, 2004; Yu *et al.*, 2005). Sterigmatocystin (ST) or dihydrosterigmatocystin (DHST), the penultimate precursors of aflatoxins, are produced by several species including *Aspergillus versicolor* and *Aspergillus nidulans*. ST and DHST are toxic and carcinogenic as well. They share common biochemical pathways, homologous genes, and regulatory mechanisms to aflatoxin synthesis in *A. flavus* and *A. parasiticus* (Brown *et al.*, 1996; Yu *et al.*, 2004a). The ST biosynthetic pathway and genes are also discussed below where appropriate.

Aflatoxins are synthesized from malonyl CoA, first with the formation of hexanoyl CoA, followed by formation of a decaketide anthraquinone (Bhatnagar *et al.*, 1992; Minto and Townsend, 1997). There are two fatty acid synthases (FAS) and a polyketide synthase (PKS) involved in the synthesis of the polyketide from acetyl CoA (Watanabe and Townsend, 2002). Norsolorinic acid (NOR) is the first stable aflatoxin intermediate identified in the pathway (Bennett *et al.*, 1981; Bennett *et al.*, 1997). Aflatoxins are formed after a series of highly organized oxidation-reduction reactions (Bhatnagar *et al.*, 1992; Townsend, 1997; Yabe *et al.*, 2003; Yu *et al.*, 2004c). The general accepted aflatoxin biosynthetic pathway scheme is: a hexanoyl CoA precursor → norsolorinic acid, NOR → averantin, AVN → hydroxyaverantin, HAVN → Oxoaverantin, OAVN → averufin, AVF → hydroxyversicolorone, HVN → versiconal hemiacetal acetate, VHA → versiconal, VAL → versicolorin B, VERB → versicolorin A, VERA → demethyl-sterigmatocystin, DMST → sterigmatocystin, ST → O-methylsterigmatocystin, OMST → aflatoxin B_1, AFB_1 and aflatoxin G_1, AFG_1. After the VHA step, there is a branch point in the pathway that leads to AFB_1 and AFG_1 formation, as well as AFB_2 and AFG_2 (Yu *et al.*, 1998; Yabe *et al.*, 2003).

A total of 29 genes or open reading frames (ORFs) are involved in aflatoxin formation (Yu *et al.*, 2004c). These genes have been cloned and most have been characterized (Yu *et al.*, 2004a; Yu *et al.*, 2004c). The first aflatoxin pathway gene *aflD* (*nor-1*), was identified by complementation studies that selected for the characterisitic red color of norsolorinic acid. This gene encodes for a ketoreductase in *A. parasiticus* for the conversion of norsolorinic acid (NOR) to averantin (AVN)(Chang *et al.*, 1992; Bennett *et al.*, 1997). Disruption or deletion of the *aflD* (*nor-1*) gene lead to the accumulation of a -red pigment in the hyphae and partially blocked the synthesis of all aflatoxins and their intermediates beyond NOR (Lee *et al.*, 1971; Bennett *et al.*, 1971; Bennett *et al.*, 1981). The

aflM (*ver-1*) gene, encoding for a ketoreductase required for the conversion of versicolorin A (VERA) to demethylsterigmatocystin (DMST) and versicolorin B (VERB) to demethyldihydrosterigmatocystin (DMDHST) was the second gene cloned in *A. parasiticus* (Liang *et al.*, 1996) (Skory *et al.*, 1992; Skory *et al.*, 1993). The third gene named *aflP* (*omtA*) encoding an *O*-methyl-transferase for conversion of sterigmatocystin (ST) to *O*-methylster-igmatocystin (OMST) and demethylsteri-gmatocystin (DMST) to dihydro-*O*-methylsterigmatocystin (DHOMST) was cloned by antibody screening of a cDNA expression library from *A. parasiticus* (Yu *et al.*, 1993). A regulatory gene named *aflR*, present in *A. parasiticus* and *A. flavus*, as well as in *A. nidulans* (originally named *afl-2* and *apa-2*), was also cloned shortly thereafter (Chang *et al.*, 1993; Payne *et al.*, 1993). This is a positive regulatory gene involved in both aflatoxin pathway gene expression in *A. flavus* and *A. parasiticus* and sterigmatocystin (ST) pathway gene expression in *A. nidulans*. The identification of these four genes for aflatoxin or ST biosynthesis were milestone discoveries that lead to the identification of the aflatoxin gene cluster by matching overlapping cosmid clones.

Genes involved in the early stages of aflatoxin biosynthesis include two large genes (7.5-kb transcripts), *aflB* (*fas-1*) and *aflA* (*fas-2*), encoding beta (FASβ) and alpha-subunits (FASα) of a fatty acid synthase, respectively (Watanabe *et al.*, 1996; Trail *et al.*, 1995a; Mahanti *et al.*, 1996). Another large gene in aflatoxin synthesis is the 7 kb *aflC* (*pksA*) gene encoding a polyketide synthase (PKS) for the synthesis of the polyketide skeleton (Chang *et al.*, 1995b; Watanabe *et al.*, 1996). Disruption of the *aflC* (*pksA*) gene results in non-production of aflatoxin or any of its intermediates (Feng and Leonard, 1995). The predicted amino acid sequence of this PKS reveals four typical conserved domains common to other known PKS proteins: beta-ketoacyl synthase (KS), acyltransferase (AT), acyl carrier protein (ACP), and thioesterase (TE) (Chang *et al.*, 1995b). To recapitulate, *aflA*, *aflB*, *aflC* genes are directly involved in the conversion of acetate to norsolorinic acid (NOR).

The cloning of *aflQ* (*ordA*) and *aflU* (*cypA*) solved the major puzzle in the latter stages of aflatoxin biosynthesis especially for the synthesis of the G-group aflatoxins (Yu *et al.*, 1998; Ehrlich *et al.*, 2004). There are two separate pathways leading to B-Group (AFB_1 and AFB_2) and G-Group (AFG_1 and AFG_2) aflatoxins (Yabe *et al.*, 1988). The gene named *aflQ* (*ordA*), encoding a cytochrome P-450 monooxygenase, was demonstrated to be responsible for the conversion of *O*-methylsterig-matocystin (OMST) to AFB_1 and AFG_1, and demethyldihydrost-erigmatocystin (DMDHST) to AFB_2 and AFG_2 (Prieto and Woloshuk, 1997; Yu *et al.*, 1998) in *A. parasiticus* and in *A. flavus*. Expression and substrate feeding, using a yeast system, demonstrated that an additional enzyme was required for formation of G-group of aflatoxins (AFG_1 and AFG_2) (Yu *et al.*, 1998). Functional studies demonstrated that the *aflU* (*cypA*) gene in *A. parasiticus*, encoding a cytochrome P450 monooxygenase, was responsible for the conversion of OMST to AFG_1 and DHOMST to AFG_2 (Ehrlich *et al.*, 2004). The inability to produce G-group aflatoxins in *A. flavus* is thought to be due to a

partial deletion of this gene during the evolution of *A. flavus* (Ehrlich *et al.*, 2004).

Aflatoxin biosynthetic pathway gene cluster

The *aflD* (*nor-1*) and *aflM* (*ver-1*) genes were found to be linked with the regulatory gene *aflR* in a common cosmid clone (Skory *et al.*, 1993; Trail *et al.*, 1995b). This provided the initial evidence indicating that aflatoxin pathway genes were clustered. The aflatoxin pathway gene cluster was established when 9 cloned genes, including *aflD* (*nor-1*), *aflR*, *aflM* (*ver-1*), and *aflP* (*omtA*), were mapped to within 75 kb DNA region by overlapping cosmid clones in *A. parasiticus* and *A. flavus* (Yu *et al.*, 1995). The completed aflatoxin pathway gene cluster was established when an 82 kb DNA sequence harboring a total of 29 aflatoxin biosynthetic pathway genes (or ORFs) and 4 sugar utilization genes was reported (Yu *et al.*, 2004c). The primary evolutionary advantage of gene clustering may be for the purpose of coordinated gene expression. Interestingly, a partially duplicated aflatoxin gene cluster was identified, in *A. parasiticus*, (Liang *et al.*, 1996). The duplicated gene cluster consists of seven duplicated genes, named *aflR2*, *aflJ2*, *adhA2*, *estA2*, *norA2*, *ver1B*, and *omtB2* respectively (Chang and Yu, 2002). The number "2" denotes the second copy of the gene. The genes within this partially duplicated cluster are likely non-functional under normal conditions, although some of the gene sequences are intact. Lack of expression may be due to the chromosome location (Chiou *et al.*, 2002), but this remains uncharacterized.

Genetic regulation of aflatoxin biosynthesis

The *aflR* gene is a positive regulatory gene in both the aflatoxin and sterigmatocystin gene clusters. This gene activates toxin pathway gene transcription (Chang *et al.*, 1993; Payne *et al.*, 1993). Disruption of *aflR* results in the loss of aflatoxin pathway gene expression and aflatoxin production. Inclusion of an additional copy of *aflR* or elevated expression leads to overproduction of aflatoxin biosynthetic intermediates (Chang *et al.*, 1995c; Flaherty and Payne, 1997). The *aflR* gene, coding for a sequence specific zinc binuclear DNA-binding protein, is required for transcriptional activation of most, if not all, of the aflatoxin pathway genes (Woloshuk *et al.*, 1994; Chang *et al.*, 1995c; Chang *et al.*, 1999b; Chang *et al.*, 1999a). The AflR protein has major domains typical of fungal and yeast Gal4-type transcription factors (Chang *et al.*, 1995c): a N-terminal cysteine-rich stretch, (Cys_6-Zn_2) DNA-binding domain (Woloshuk *et al.*, 1994; Chang *et al.*, 1995c) an arginine-rich (RRARK) nuclear localization domain; and a transcription activation domain in the C-terminus (Chang *et al.*, 1999a; Chang *et al.*, 1999b). Aflatoxin pathway gene transcription is activated when the AflR protein binds to the palindromic sequence 5'-TCGN5CGA-3' (also called AflR binding motif) in the promoter region of structural genes (Fernandes *et al.*, 1998; Ehrlich *et al.*, 1999b; Ehrlich *et al.*, 1999a) in *A. parasiticus, A. flavus* and *A. nidulans*. *A. sojae*, a non-toxigenic species used in industrial

fermentations, was found to contain a defective *aflR* transcription activation domain due to early termination of 62 amino acids from its C-terminus (Matsushima *et al.*, 2001; Takahashi *et al.*, 2002). Thus, with the absence of the functional regulatory protein, no induction of aflatoxin can occur in this food grade *Aspergillus*. Adjacent to the *aflR* gene in the aflatoxin gene cluster, a divergently transcribed gene, *aflS* (*aflJ*), was also found to be involved in the regulation of transcription (Meyers *et al.*, 1998). The AflJ protein binds to the carboxy terminal region of AflR and may affect AflR activity (Chang, 2003). Disruption of *aflS* in *A. flavus* resulted in a failure to produce any aflatoxin pathway metabolites (Meyers *et al.*, 1998).

An important newly described regulatory gene named *laeA*, (named such for lack of *aflR* expression), was identified to reside outside of the aflatoxin pathway gene cluster (Butchko *et al.*, 1999; Bok and Keller, 2004). Disruption of *laeA* resulted in loss not only of *aflR* gene expression for ST synthesis, but also expression of genes involved in penicillin biosynthesis in *A. nidulans*, as well as genes involved in gliotoxin biosynthesis in *A. fumigatus* (Bok and Keller, 2004). Thus it is becoming apparent that *laeA* appears to be involved in the global regulation in the biosynthesis of a number of different secondary metabolites in several fungal species.

Biological regulation of aflatoxin biosynthesis

Aflatoxin biosynthesis is influenced by many biotic and abiotic factors, including nutritional factors such as carbon or nitrogen source; environmental effects such as water activity and temperature; physiological conditions such as pH; and bioreactive agents such as plant and environmental chemicals. These non-genetic parameters affect the physiology of aflatoxigenic molds in various ways, thus these non-genetic factors can be considered as environmental or biological regulation of aflatoxin formation. Some of these factors may work to change expression of the aflatoxin regulatory gene, *aflR*, or alter the expression of globally acting transcription factors that respond to external signals. To date, the molecular mechanisms that mediate these effects are not clear (Payne and Brown, 1998; Guo *et al.*, 2005), the subject matter is under intensive study. These studies offer promise of devising control strategies to shut down aflatoxin production in aflatoxigenic *A. flavus* species through manipulations of environmental conditions of the fungal response to these factors.

Nutritional factors such as carbon, nitrogen, amino acid, lipid, and trace elements have long been observed to affect aflatoxin production (Payne and Brown, 1998; Feng and Leonard, 1998; Cuero *et al.*, 2003). The relationship of carbon source and aflatoxin formation has been well established with simple sugars such as glucose, sucrose, maltose (but not peptone, sorbose, or lactose) supporting aflatoxin formation (Payne and Brown, 1998). However, the molecular mechanism by which a carbon source is involved in the regulation of aflatoxin pathway gene expression is poorly understood. Nitrogen source also affects aflatoxin formation in varying ways (Payne and Brown, 1998). Aflatoxin

production ceases on nitrate but not on ammonium containing media. Certain amino acids can have opposing effects on aflatoxin production (Payne and Hagler, 1983). Recent studies show that tryptophan inhibits aflatoxin formation while tyrosine enhances aflatoxin production in *A. flavus* (Wilkinson *et al.*, 2007b). Micronutrients (metal ions) also affect aflatoxin pathway gene expression (Bennett *et al.*, 1979; Cuero *et al.*, 2003). Lipids have tremendous effects on aflatoxin formation, not only as a nutritive source but as substrates metabolized for acyl-CoA starter units (Maggio-Hall *et al.*, 2005) and as signaling molecules (Yu *et al.*, 2003; Brodhagen and Keller, 2006).

Temperature, water activity (drought stress) and other stress elements are external environmental factors that affect aflatoxin production (Cotty, 1988; Keller *et al.*, 1997; Payne and Brown, 1998; Guo *et al.*, 2005; Kim *et al.*, 2005; Kim *et al.*, 2006; OBrian *et al.*, 2007) Studies suggest that *aflR* transcription is responsive to a G-protein signaling cascade that is mediated by protein kinase A (Hicks *et al.*, 1997) (discussed later). The signaling pathway may respond to external environmental signals and then mediate effects on aflatoxin biosynthesis. Optimal aflatoxin production is observed at temperatures near 30°C (28°C to 35°C) (OBrian *et al.*, 2007). When temperature increases to above 36°C, aflatoxin production in the fungus is nearly completely inhibited. This is associated with a decrease in the expression of the aflatoxin pathway genes (OBrian *et al.*, 2007). Interestingly, temperature does not appear to significantly affect the expression of the regulatory genes *aflR* or *aflS* (OBrian *et al.*, 2007). This may indicate that temperature affects the activity of AflR or some other unknown regulatory element.

Ambient pH and other environmental conditions are also important factors affecting aflatoxin formation (Cotty, 1988). Aflatoxin biosynthesis in *A. flavus* occurs in acidic media, but is inhibited in alkaline media (Cotty, 1988; Price *et al.*, 2005). The presence of a putative PacC-binding site close to the *aflR* transcription start site may play some role in pH regulation on aflatoxin production (Tilburn *et al.*, 1995; Keller *et al.*, 1997) and the PacC and AreA (Chang *et al.*, 2000) binding sites in the *aflR-aflS* (*aflJ*) intergenic region suggest that gene expression is regulated by environmental signals (pH & nitrate).

Many fungal developmental stages are associated with secondary metabolism such as sporulation and sclerotial formation (Bennett *et al.*, 1986; Hicks *et al.*, 1997; Chang *et al.*, 2002; Calvo *et al.*, 2002), for example similar environmental conditions are required for secondary metabolism and sporulation with spore formation, and secondary metabolite formation occurs at about the same time (Trail *et al.*, 1995b; Hicks *et al.*, 1997). Some mutants that are deficient in sporulation are unable to produce aflatoxins (Bennett and Papa, 1988) and some compounds that inhibit sporulation in *A. parasiticus* also inhibit aflatoxin formation (Reiss, 1982). Moreover, chemicals that inhibit polyamine biosynthesis in *A. parasiticus* and *A. nidulans* inhibit both sporulation and aflatoxin/ST biosynthesis (Guzman-de-Pena *et al.*, 1998). A more recent finding reveals that the regulation of sporulation and ST production is through a shared

G-protein mediated growth pathway in *A. nidulans* (Hicks *et al.*, 1997; Yu and Keller, 2005). Mutations in *A. nidulans flbA* and *fadA* genes, early acting members of a G-protein signal transduction pathway, result in loss of ST gene expression, ST production, and sporulation (Yu *et al.*, 1996; Hicks *et al.*, 1997). The regulation is partially mediated through protein kinase A (Shimizu and Keller, 2001). This G-protein signaling pathway involving FadA in the regulation of aflatoxin production also exists in *A. parasiticus* and *A. flavus* (Hicks *et al.*, 1997).

The relationship of oxidative stress and aflatoxin biosynthesis in *A. parasiticus* has long been reported (Jayashree and Subramanyam, 2000; Mahoney and Molyneux, 2004; Reverberi *et al.*, 2006; Kim *et al.*, 2006). Jayashree *et al.* (2000) were the first to report that oxidative stress induced aflatoxin biosynthesis in *A. parasiticus*. Kim *et al.* (2006) showed that treatment of *A. flavus* with *tert*-butyl hydroperoxide, induced significant increases in aflatoxin production. Similar treatment of *A. parasiticus* also induced aflatoxin production (Reverberi *et al.*, 2005; Reverberi *et al.*, 2006). Hydrolysable tannins significantly inhibit aflatoxin biosynthesis, with the main anti-aflatoxigenic constituents in these tannins being gallic acid (Mahoney and Molyneux, 2004). Gallic acid reduces expression of structural genes within the aflatoxin biosynthetic cluster, but surprisingly not the aflatoxin pathway gene regulator, *aflR*. It appears that gallic acid disrupts signal transduction pathway(s) for aflatoxigenesis somewhere upstream of the gene cluster. When certain phenolics or other antioxidants, such as ascorbic acid, are added to oxidatively stressed *A. flavus*, aflatoxin production significantly declines, with no effect on fungal growth (Kim *et al.*, 2006, 2008). Caffeic acid is another antioxidant that inhibits aflatoxigenesis. Microarray analysis of *A. flavus* treated with caffeic acid identified a gene, named *ahpC2*, an alkyl hydroperoxide reductase that is potentially involved in quelling the signal for aflatoxin production. However, no notable effect on expression of *laeA*, a gene encoding a global regulator for secondary metabolism in *Aspergillus* (Bok and Keller, 2004) was observed when under caffeic acid treatment. It is becoming obvious that many different regulatory mechiams affect regulation of aflatoxin production and other secondary metabolites.

Genomics of *Aspergillus flavus*

Genomics is the process of revealing the entire genetic contents of an organism, by high throughput sequencing of the DNA and bioinformatics identification of all of the open reading frames (ORFs). Recent technological breakthroughs allow scientists to sequence and annotate genomes in a very short time frame. A combination of Expressed Sequence Tags (EST), whole genome sequencing, and microarray technologies provide high throughput capabilities (Bennett and Arnold, 2001; Yu *et al.*, 2004d; Kim *et al.*, 2006; Payne *et al.*, 2006) that can be applied to the identification of genes involved in aflatoxin production and for studying the regulatory mechanisms of gene expression (*i.e.*, functional genomics).

Aspergillus flavus expressed sequence tags

An *A. flavus* EST project was completed using the wild type strain NRRL 3357 (ATCC# 20026). Over 26,110 cDNA clones from a normalized cDNA expression library were sequenced at The Institute for Genomic Research (TIGR). A total of 19,618 *A. flavus* ESTs were generated, from which 7218 unique EST sequences were identified (Yu *et al.*, 2004d). These EST sequences have been released to the public at the NCBI GenBank Database (http://www.ncbi.nlm.nih.gov/). The *A. flavus* Gene Index was constructed at TIGR (http://www.tigr.org) which is currently maintained and curated by The Dana Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi). From the EST database, an additional four new transcripts (*hypB*, *hypC*, *hypD*, and *hypE*) were identified in the aflatoxin biosynthetic gene cluster, which were not identified during chromosomal walking. In addition, several categories of other genes identified could potentially be involved, directly or indirectly, in aflatoxin production, such as in global regulation, signal transduction, pathogenicity, virulence, and fungal development (Yu *et al.*, 2004d).

Whole genome sequencing of *Aspergillus flavus*

The *A. flavus* whole genome sequencing project was funded by a USDA, National Research Initiative grant awarded to Professor Gary A. Payne and Ralph Dean, North Carolina State University, Raleigh, North Carolina. The Food and Feed Safety Research Unit of Southern Regional Research Center, USDA/ARS, provided funding for fine finishing and gene calling. The sequencing was completed at The Institute for Genmic Research (TIGR) under the supervision of Dr. William C. Nierman by a shotgun approach and Sanger sequencing protocol. Primary assembly indicated that the *A. flavus* genome consists of 8 chromosomes and the genome size is about 36.8 Mb. Aided with the *A. flavus* EST database, the *A. oryzae* EST database, and the *A. oryzae* whole genome sequence, annotation of the *A. flavus* genome sequence data have been almost completed. Preliminary results demonstrate that there are over 12,000 functional genes in the *A. flavus* genome, a number similar to those of other *Aspergillus* species (Nierman *et al.*, 2005; Machida *et al.*, 2005; Galagan *et al.*, 2005; Payne *et al.*, 2006; Yu and Cleveland, 2007). Genes responsible for the biosynthesis of secondary metabolites, such as aflatoxins, have been identified and include those encoding PKSs, non-ribosomal peptide synthethases (NRPS), cytochrome P450 monooxygenases, fatty acid synthases (FAS), carboxylases, dehydrogenases, reductases, oxidases, oxidoreductases, epoxide hydrolases, oxygenases, and methyltransferases (Yu *et al.*, 2004c). The availability of the *A. oryzae* whole genome sequence (Machida *et al.*, 2005) provided not only the sequence data but the chromosomal structure for comparison with *A. flavus*. The sequence data have been deposited with the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) and are also available through the *Aspergillus flavus* website (http://www.aspergillusflavus.org).

Microarrays as tools for functional genomics studies

Several types of microarrays have been constructed within the last few years. The first cDNA amplicon microarray, consisting of 753 gene features, including known aflatoxin pathway genes *aflD* (*nor-1*) and *aflP* (*omtA*) and regulatory gene *aflR*, was constructed by the laboratory of Gary Payne, North Carolina State University. The unique ESTs identified from a cDNA library constructed using *A. flavus* RNA under aflatoxin-producing condition were spotted on Telechem SuperAldehyde glass slide using an Affymetrix 417 Arrayer (OBrian *et al.*, 2003). A 5,002 gene-elements *A. flavus* EST based amplicon microarray was constructed at TIGR by the Food and Feed Safety Research Unit, USDA/ARS, Southern Regional Research Center. Using genomic DNA as template, the specific gene sequences were amplified using primers synthesized based on *A. flavus* unique EST sequences assembled. This microarray has been updated to a 5031 gene-element array including genes of interest when their sequences became available.

A comprehensive whole genome *A. flavus* oligo microarray has also been constructed at TIGR by the Food and Feed Safety Research Unit, USDA/ARS, Southern Regional Research Center. All of the 11,820 *A. flavus* unique genes, the unique genes present in *A. oryzae* but absent in *A. flavus*, and 10 genes cloned from corn that show resistance against *A. flavus* infection, have been represented by this whole genome microarray. An additional Affymetrix GeneChip microarray funded by a grant from USDA/NRI awarded to a Consortium led by Gary Payne was designed and constructed by Affymetrix Inc. This Affymetrix array contains all of the *A. flavus* genes, *A. oryzae* unique genes, plus additional genes of interest from corn, *Fusarium* species, mouse and human genomes. Additionally, a peanut/*A. flavus* combined microarray, funded by Crop Protection and Management Laboratory, USDA/ARS, Tifton Georgia, is under construction at J. Craig Venter Institute (JCVI), the not for profit organization previously named The Institute for Genomic Research (TIGR). This crop/fungus combined array contains oligos representing over 10,000 peanut ESTs, and all of the annotated *A. flavus* and *A. oryzae* unique genes. Profiling of genes involved in aflatoxin formation using these microarrays, performed at USDA labs, the labs of North Carolina State University, TIGR and JCVI, identified hundreds of genes that are significantly up or down regulated under various growth conditions of the fungus (OBrian *et al.*, 2003; Price *et al.*, 2005; Kim *et al.*, 2006, 2008; Price *et al.*, 2006; OBrian *et al.*, 2007; Chang *et al.*, 2007; Wilkinson *et al.*, 2007a; Yu *et al.*, 2007b; Cary *et al.*, 2007). Further studies using these microarray resources for a genome-wide gene profiling and functional analysis in relation to aflatoxin formation will surely reveal the processes that triggers aflatoxin production and the regulation of the process. The knowledge will empower researchers to find effective strategies for controlling aflatoxin contamination of food and feed.

Future perspectives

Over 40 years of research and investigation have generated a wealth of published information of fungal biology, toxicology and aflatoxin biosynthesis. We now have a good understanding of the genetics of aflatoxin biosynthesis in *A. flavus* through studies by traditional genetic methods, modern genetic cloning techniques, and use of high throughput genomic technologies. We have described the aflatoxin pathway and the pathway genes, and we have shown that these genes reside in a cluster as do genes for sugar utilization. With the rapid progress in fungal genomics, we will master a vast amount of new information on gene function, genetic regulation and signal transduction within these fungal systems as well as their interactions with the environment. Several types of microarrays with different formats are now readily available for these purposes. The genetic and genomic resources will significantly enhance our understanding of the mechanisms of aflatoxin production, pathogenicity of the fungus, and crop-fungus interactions. The results stemming from research on *A. flavus* genomics are expected to provide valuable information for devising novel strategies to eliminate aflatoxin contamination resulting in a safer, nutritious and sustainable food and feed supply.

Conclusion

With the advancement in technological development, the wealth of knowledge on aflatoxin formation, and the *Aspergillus flavus* genome data, we will be able to better understand the mechanisms of genetic and biological regulation of aflatoxin production. This information is vital for the reduction and even elimination of aflatoxin contamination in food and feed. On the other hand, fully understanding the genetics and genomics of *Aspergillus flavus* can turn the detrimental fungus into a beneficial one. There are many secondary metabolites produced by *A. flavus* that could be explored for potential use as pharmaceuticals or industrial applications. The cellulosic enzymes from the fungus can be used for bioenergy production through industrial fermentation.

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CHAPTER - 3

GENETIC TRANSFORMATION OF ZYGOMYCETES FUNGI

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Introduction

Zygomycetes fungi, especially members of the order Mucorales and Mortierellales are important from various biological, biotechnological and medical aspects. They are used as model organisms to answer biological questions in the fields of sexual differentiation (Sutter, 1975; Kuzina and Cerdá-Olmedo, 2006; Idnurm *et al.,* 2008), morphological dimorphism (Lübbehüsen *et al.,* 2003; Iturriaga *et al.,* 2005), biosynthesis of carotenoids (Iturriaga *et al.,* 2000; Sanz *et al.,* 2002; Kuzina *et al.,* 2006; Almeida and Cerdá-Olmedo, 2008) or the light induction of gene regulation and metabolite production (Velayos *et al.,* 2003; Quiles-Rosillo *et al.,* 2005; Idnurm *et al.,* 2006). *Phycomyces blakesleeanus*, *Mucor circinelloides*, *M. mucedo*, *Rhizopus oryzae* and *Absidia glauca* are the best-studied species. *Rhizopus*, *Mucor* and *Gilbertella* species may also be important causing post-harvest losses in agricultural products, or as spoilage microorganisms of certain foods (Csernetics *et al.,* 2005). Several species belonging to the genera *Rhizopus*, *Absidia*, *Rhizomucor*, *Mucor*, *Apophysomyces*, *Saksanea*, *Cunninghamella* , *Cokeromyces* and *Syncephalastrum* are known as causative agents of frequently fatal opportunistic fungal infections in immunocompromised patients called as zygomycoses (Ribes *et al.,* 2000;

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Chayakulkeeree *et al.*, 2006; Papp *et al.*, 2008). High mortality rates, difficulties in the diagnosis and non-treatability with the most widely used antifungal drugs are characteristic features of such infections. Studies on the molecular and genetic background of the pathogenicity of these fungi started only a few years ago (Papp *et al.*, 2008).

Recently, there is an increasing interest in the exploitation of the biotechnological potential of zygomycetes fungi. They are used as bio-transforming agents in steroid production and as producers of hydrolytic enzymes, organic acids and other valuable bioactive metabolites, such as carotenoids or polyunsaturated fatty acids. Most significant species are *R. oryzae* used for lactic acid production (Abe *et al.*, 2003; John *et al.*, 2007), *Rhizomucor miehei* producing rennin-like aspartyl protease (Outtrup and Boyce, 1990; Rao *et al.*, 1998), *Mortierella alpina* (Shinmen *et al.*, 1989) used for production of arachidonic acid and other polyunsaturated fatty acids and *Blakeslea trispora*, which is an industrial source of β-carotene and lycopene (Mehta *et al.*, 2003; Lopez-Nieto *et al.*, 2004). Oriental soybean-based food fermentations (e.g. production of tempeh and sufu) also involve some zygomycetes, like *Rhizopus oligosporus* (Hachmeister and Fung, 1993).

Biological investigations, pathogenicity studies and biotechnological developments require routinely applicable methods and useful tools for functional gene analysis and manipulation including appropriate selection and transformation procedures, well characterized molecular markers and expression signals. During the past decade, molecular genetic studies on this fungal group have been spectacularly progressed in all above-mentioned fields of research. Recently, the genome sequences of three Zygomycetes model organis- ms (*R. oryzae*: http://www.broad.mit.edu/annotation/genome/ rhizopus_oryzae/Home. html, *P. blakesleeanus*: http://genome.jgi-psf.org/Phybl1/ Phybl1.home.html and *M. circinelloides*: http://mucorgen.um.es/) have been completed, which will substantially facilitate the further exploration of knowledge. Considering all these aspects, a point has been reached where it seems to be useful to summarize and review the *state*-of-the-*art methods* for genetic manipulation of Zygomycetes.

Selection methods

Two types of selectable markers are used in transformation studies on zygomycetes: dominant selection markers based on drug resistance or utilization of a specific substrate and auxotrophic markers. A prerequisite for the usage of auxotrophy complementation is the availability of a stable mutant from the strain desired to transform, whereas dominant selection methods allow direct transformation of wild-type strains without the need of a previous mutagenesis. Table 3.1 contains an overview of the selectable markers which have been used for the transformation of different fungi belonging to the zygomycetes.

Table 3.1. Selection markers applied for the transformation of zygomycetes fungi

Marker[a]	Encoded function	Origin of the gene	Transformed species	Reference
Dominant markers				
Drug resistance				
hph	hygromycin B phosphotransferase	*Escherichia coli*	*Backusella lamprospora*	Nyilasi *et al.*, 2008
			Mucor circinelloides	Nyilasi *et al.*, 2005
hpt	hygromycin B phosphotransferase	*Escherichia coli*	*Mortierella alpina*	Mackenzie *et al.*, 2000
*aph*I	aminoglycoside phosphotransferase	*Escherichia coli*	*Rhizomucor miehei*	Monfort *et al.*, 2003
*npt*I	neomycin phospho-transferase	*Escherichia coli*	*Phycomyces blakesleeanus*	Obraztsova *et al.*, 2004
*npt*II	neomycin phospho-transferase	*Escherichia coli*	*Absidia glauca*	Wöstemeyer *et al.*, 1987
			Parasitella simplex	Burmester, 1992
kan	kanamycin resistance	*Escherichia coli*	*Mucor circinelloides*	Appel *et al.*, 2004
			Mucor rouxii	Appel *et al.*, 2004
			Rhizomucor pusillus	Appel *et al.*, 2004
			Phycomyces blakesleeanus	Arnau *et al.*, 1988
G418^{r}	geneticin resistance	*Escherichia coli*	*Rhizopus niveus*	Yanai *et al.*, 1990
cbx^{R}	carboxin resitance	*Ustilago maydis*	*Mucor circinelloides*	Ortiz-Alvarado *et al.*, 2006
Zeo	zeocin resistance	pEM7/Zeo, Invirogen	*Mortierella alpina*	Takeno *et al.*, 2005
Specific substrate utilization				
amdS	acetamidase	*Aspergillus nidulans*	*Rhizopus oryzae*	Michielse *et al.*, 2004
Auxotrophic markers				
leuA (*leu1*)	α-isopropylmalate dehydrogenase	*Mucor circinelloides*	*Mucor circinelloides*	van Heeswijk and Roncero, 1984
			Rhizopus niveus	Liou *et al.*, 1992
		Phycomyces blakesleeanus	*Mucor circinelloides*	Iturriaga *et al.*, 1992
		Rhizomucor pusillus	*Rhizomucor pusillus*	Wada *et al.*, 1996
		Rhizopus niveus	*Rhizopus niveus*	Takaya *et al.*, 1996
met+	unknown	*Mucor circinelloides*	*Mucor circinelloides*	Anaya and Roncero, 1991
pyrF (*ura5*)	orotate phosphoribosyl-transferase	*Mortierella alpina*	*Mortierella alpina*	Takeno *et al.*, 2004
		Rhizopus oryzae	*Rhizopus oryzae*	Ibrahim *et al.*, 2007b

pyrG (*pyr4*)	orotidine-5-monophosphate decarboxylase	*Blakeslea trispora*	*Mucor circinelloides*	Quiles-Rosillo *et al.*, 2003[a]
		Mucor circinelloides	*Mucor circinelloides*	Benito *et al.*, 1992
			Rhizomucor miehei	Lukács *et al.*, 2009
		Rhizomucor pusillus	*Rhizomucor pusillus*	Yamasaki *et al.*, 1999
		Rhizopus niveus	*Rhizopus delamar*	Horiuchi *et al.*, 1995
			Rhizopus oryzae	Michielse *et al.*, 2004
		Rhizopus oryzae	*Rhizopus oryzae*	Skory, 2002

[a]Gene designations are presented as published in the original works

Dominant selection methods

Drug resistance markers are genes (most commonly of bacterial origin) providing resistance for the fungus to an appropriate drug present in the selection medium. Unfortunately, zygomycetes are known to be poorly susceptible to the majority of the commonly used compounds. Earlier, *M. circinelloides* was reported to be resistant to hygromycin B, geneticin, neomycin, oligomycin and benomyl (van Heeswijck *et al.*, 1988). Different strains of the same species may differ in their susceptibility to the commonly used drugs. For example, Mackenzie *et al.* (2000) found only one hygromycin B sensitive strain among the six *Mo. alpina* strains tested. Later, Takeno *et al.* (2004) did not find any antibiotic or inhibitor which were applicable in transformation experiments of the arachidonic acid producer *Mo. alpina* 1S-4 strain.

In some cases, the susceptibility of the fungus could be increased by adjustment of the medium pH, selection in liquid medium or addition of specific compounds to the selection medium (Yanai *et al.*, 1990; Ibrahim and Skory, 2007a). Recently, Nyilasi *et al.* (2005) reported that susceptibility of *Mucor* to hygromycin B could be increased by the addition of Rose Bengal and dichloran to the culture medium. Fungal growth was completely blocked at ≥50 μg/ml hygromycin B in the presence of 3 μg/ml dichloran and 100 μg/ml Rose Bengal. In spite of these difficulties, there are some examples for more or less successful application of such markers, most frequently hygromycin B phosphotransferase and Tn5- or Tn903-derived kanamycin resistance genes (Table 3.1).

Applications of genes providing the ability to utilize specific substrates are not well established for zygomycetes. Michielse *et al.* (2004) compared the efficacy of the *Aspergillus nidulans amdS* gene as a dominant marker with that of the auxotrophic marker *pyr4* for *R. oryzae* (*amdS* encodes the acetamidase enzyme that enables fungi to utilize acetamide as a sole nitrogen source). They used a *pyr4*/*amdS* double selection vector and performed primary selection for the *pyr4* marker. Among the *pyr4*$^+$ transformants only a few proved to be *amdS*$^+$; these strains contained autonomously replicating vector molecules with a very low mitotic stability. It is worth to mention, that such genes often prove to be weak markers of the transformation event, because majority of zygomycetes are

able to grow on nutritionally restricted media. For example, in our tests, *Gilbertella persicaria*, *M. hiemalis*, *R. microsporus*, *R. oryzae* and *Rm. miehei* showed slight growth on different minimal media containing acetamide as the sole nitrogen source (unpublished results). The possibility that the untransformed strains may produce a "background-growth" on the selection medium makes largely inconvenient the application of substrate utilization based markers.

Auxotrophic markers

Majority of transformation systems developed for fungi belonging to the zygomycetes involve auxotrophy complementation to select for the transformants. Most frequently used selectable markers are the α-isopropylmalate dehydrogenase (*leuA* or *leu1*) and the orotidine-5-monophosphate decarboxylase (*pyrG* or *pyr4*) genes complementing leucine and uracil auxotrophy, respectively. For *Mo. alpina* an overall transformation system was developed recently, where the orotate phosphoribosyl transferase gene (*ura5* or *pyrF*) complementing uracil auxotrophy was used as selection marker (Takeno *et al.,* 2004). Ibrahim *et al.* (2007b) also used *pyrF* selection in expression analysis of the *Rhizopus* high-affinity iron permease 1 gene.

Auxotrophic mutants of zygomycetous fungi are generally created by UV or chemical mutagenesis. Such mutant strains must be examined before the transformation experiments to exclude other possible mutations that change the physiological characteristics of the fungus.

Transformation procedures

Polyethylene glycol (PEG) mediated protoplast transformation

Traditionally, the most common methodology for zygomycetes is the PEG-mediated transformation. This technique requires protoplast formation prior to the transformation when cells are treated with cell wall-degrading enzymes in a buffer that provides an osmotically stable environment. There are *strain to strain* differences with respect to the composition and amount of the digesting enzyme mixture as well as the concentration of the osmotic buffers have to be used in these experiments. According to the cell wall composition of zygomycetes, wall-degrading enzyme mixtures generally contain chitinase and chitosanase. These protoplast forming enzymes can be purchased from a commercial source or can be prepared via culturing *Streptomyces* on purified cell wall of the appropriate zygomycetous strain ("Streptozym"; Suárez *et al.,* 1987). In our laboratory, snail enzyme (*Helix pomatia* gastric juice) in a concentration of about 1.5% has also been used routinely to digest the cell wall of Mucoralean fungi (Somogyvári *et al.,* 1996; Nyilasi *et al.,* 2008). Sporangiospores are highly resistant to lysing enzymes, thus germinating spores or young hyphae are used for protoplast formation. Germlings are generally obtained after incubation of spores in a complete liquid medium for 3 to 6 h, depending on the species and the strain

(Iturriaga *et al.,* 1992; Ibrahim and Skory, 2007a). Alternatively, spores can be inoculated onto cellophane sheets placed on solid complete medium. After culturing for 20 h at the appropriate temperature, the young colonies formed can be easily transferred into the protoplasting solution (Nagy *et al.,* 1994; Nyilasi *et al.,* 2008).

Agrobacterium tumefaciens-mediated transformation (ATMT)

A. tumefaciens is a plant pathogenic bacterium that is able to transfer a part of its DNA (T-DNA) flanked with two short direct repeats and located on a tumor-inducing plasmid (Ti plasmid) into the infected cells, where it causes the formation of crown gall tumors. The transfer depends on the expression of virulence proteins encoded by the *vir* region of the Ti-plasmid; *vir* genes can be induced by secreted compounds of wounded plant cells, such as acetosyringone. This method has the advantages that it leads to the integration of homologous or heterologous DNA fragments into the host genome, moreover, it potentially allows the transfer of relatively high molecular weight foreign DNA (at least 150 kb; Hamilton *et al.,* 1996).

ATMT is commonly used to transfer genes to a wide variety of plants and in the past decade, it has been applied for the transformation of many fungal species also; for a detailed review on the application of ATMT for fungal transformation see Michielse *et al.* (2005). The method has recently been adapted for some fungi belonging to the Zygomycetes, such as *Rm. miehei* (Monfort *et el.*, 2003), *R. oryzae* (Michielse *et al.,* 2004; Ibrahim and Skory, 2007a), *M. circinelloides* (Nyilasi *et al.,* 2005) and *B. lamprospora* (Nyilasi *et al.,* 2008).

Practically, ATMT is performed via the co-cultivation of the fungus with an *A. tumefaciens* strain harboring the appropriately modified Ti plasmid. Protocols are more or less different for all fungal species and successful transformation requires the optimization of several factors such as the quality and the amount of the fungal inoculum or the duration and temperature of the co-cultivation. Various starting materials, e.g. protoplasts, spores, germlings and even fruiting body tissues were used successfully for fungal transformation (de Groot *et al.,* 1998; Abuodeh *et al.,* 2000; Chen *et al.,* 2000; Michielse *et al.,* 2004), however, for ATMT of zygomycetes, protoplasts seem to be the most appropriate choice. Although germlings have been successfully used for the transformation of *Rm. miehei* (Monfort *et al.,* 2003) and *M. circinelloides* (Nyilasi *et al.,* 2005), in the case of *R. oryzae*, only protoplasts could be transformed with *Agrobacterium* (Michielse *et al.,* 2004). Protoplast-based approach proved to be much more efficient also for *B. lamprospora* than the direct transformation of sporangiospores (Nyilasi *et al.,* 2008). Moreover, in the latter case, co-cultivation of protoplasts or spores with the bacterium in liquid induction medium resulted in higher transformation frequency than co-cultivation on solid induction medium. For ATMT it is crucial to properly balance the bacterial and fungal growth, thus conditions of co-cultivation, especially the length of the co-cultivation period and the incubation temperature, are essential factors of an efficient transformation.

Other techniques

Although electroporation of protoplasts has been rarely applied for zygomycetes, the technique was successfully used for *A. glauca* (Schilde *et al.*, 2001): it seems to be a good alternative of the PEG-mediated method.

Another technique, the biolistic particle bombardment of sporangiospores is well established for *Mucor* (González-Hernández *et al.*, 1997), *Absidia* (Bartsch *et al.*, 2002), *Mortierella* (Takeno *et al.*, 2004) and *Rhizopus* (Skory, 2002; Ibrahim and Skory, 2007a). The main advantage of this method is the possibility to transform intact cells without the need of protoplast formation, while the need for specialized equipment and high costs have to be mentioned as drawbacks.

The fate of the introduced DNA

Autoreplicative plasmids

In spite of the efficiency of the above-described methods in providing high-frequency transformation, it is rather difficult to obtain a stable homokaryotic transformant from zygomycetes fungi. Integration of the transforming DNA into the host genome usually fails and much of the transformants prepared by the PEG-mediated procedure, electroporation or microprojectile bombardment maintain the introduced plasmids episomally (van Heeswijck and Roncero, 1984; Revuelta and Jayaram, 1986; Wöstemeyer *et al.*, 1987; Suárez and Eslava, 1988; Yanai *et al.*, 1990; Burmester, 1992; Iturriaga *et al.*, 1992; Benito *et al.*, 1995; Wolff and Arnau, 2002; Quiles-Rosillo *et al.*, 2003a; Appel *et al.*, 2004; Papp *et al.*, 2006; Ortiz-Alvarado *et al.*, 2006). Rearrangements of the transforming plasmids frequently occur in the transformants resulting in autonomously replicating nicked, broken or concatenated forms of the original plasmids (Burmester, 1992; González-Hernández *et al.*, 1997; Schilde *et al.*, 2001; Skory, 2002; Obraztsova *et al.*, 2004). This phenomenon makes difficult the molecular analysis of the transformants. Episomal plasmids and rearranged molecules have the characteristics that they do not require a defined origin for the autonomous replication; their copy number is generally low and maintenance of them is not stable because of their poor segregation into the spores (Appel *et al.*, 2004). Therefore, such transformants frequently are mitotically unstable and they may rapidly lose their plasmids under non-selective or even under selective conditions (Wolff and Arnau, 2002; Appel *et al.*, 2004; Papp *et al.*, 2006; Ortiz-Alvarado *et al.*, 2006).

Efficient selection enhances the maintenance of the plasmid. Appel *et al.* (2004) constructed a multicopy vector system for transformation of *Mucor* and *Rhizomucor* species. This vector contained two selection markers, the Tn5-derived kanamycin resistance gene and the *leuA* gene. Under selection for leucine prototrophy the plasmid copy number was very low, whereas growth on geneticin containing medium led to a remarkable increment in the copy number suggesting more efficient selection for plasmid maintenance. Unfortunately, under non-selective conditions this vector also proved to be unstable.

There are some reports on the isolation and application of sequences that promote stable autonomous replication in different zygomycetes (Roncero *et al.,* 1989; Anaya and Roncero, 1991; Benito *et al.,* 1992; Burmester *et al.,* 1992). Roncero *et al.* (1989) described an autonomous replication sequence (ARS) element in *M. circinelloides* that is situated upstream from the *leuA* gene. They found that progressing removal of sequences from this region decreased the stability of the transformants. However, in later studies, usage of the same fragment built in the transforming vectors did not result in mitotically stable transformants (Benito *et al.,* 1995; Appel *et al.,* 2004; Papp *et al.,* 2006). Burmester *et al.* (1992) isolated a fragment named as *seg1* from a neomycin resistant plasmid of *A. glauca* transformant, which proved to be stable even under non-selective conditions. This fragment originated from *A. glauca* genome and it was picked up by the transforming plasmid. In further transformation experiments, *seg1* also improved mitotic stability of the constructed plasmids (Schilde *et al.,* 2001).

Integrative systems

Autoreplicative transformation with circular plasmids can be efficient technique, for example in complementation studies or for introduction of reporter genes to analyze development-specific expression (Wöstemeyer *et al.,* 2004). However, generation of stable integrative transformants is essential for several purposes, for example, for strain improvement studies or to create null mutant strains for functional analysis of genes. In contrasts to the most filamentous fungi, achievement of the integration in zygomycetes is considered rather problematic, as mentioned earlier.

In the nineties, successful integrations were reported in *A. glauca* (Burmester *et al.,* 1990); *M. circinelloides* (Arnau *et al.,* 1991; Arnau and Stroman, 1993); *R. niveus* (Yanai *et al.,* 1990; Horiuchi *et al.,* 1995; Takaya *et al.,* 1996) and *Rm. pusillus* (Wada *et al.,* 1996; Yamazaki *et al.,* 1999). In these studies, circular plasmids were used as the transforming agent and most frequently integration occurred by additive homologous recombination; occasionally, ectopic integration and gene replacement were also observed. In some cases, the introduced DNA was found to be present not only integrated into the host genome, but also replicating episomally and rearranging with the host DNA (Yanai *et al.,* 1990; Takaya *et al.,* 1996). Some of these earlier results are difficult to explain and sometimes, misleading conclusions have been drawn from the analysis of the transformants, because rearrangements of the introduced DNA may lead to autonomously replicating, high-molecular-weight concatenated structures comigrating with the genomic DNA (Ibrahim and Skory, 2007a).

The fate of the transforming DNA has been analyzed in detail in *R. oryzae* (Skory, 2002, 2004, 2005). In these experiments, if the introduced DNA was a circular plasmid, it was replicating autonomously in concatenated arrangements;

integration at the homologous locus by additive integration or gene replacement also occurred but with very low frequency (in about 1-5% of the transformants). Cleavage of the vector sequence with one restriction enzyme had been repaired by end-joining recombination and transformation led to similar result as using a circular plasmid. When the plasmid was cleaved within a region homologous with the genomic DNA, the percentage of the additive integration events increased. If the homologous region was cut from the plasmid with two different restriction enzymes, exclusively gene replacement occurred in the transformants.

Gene replacement via double crossing-over is a commonly used approach to create gene disruption mutants for functional analysis of genes. As mentioned above, gene replacement via double crossing-over can be forced by the use of linearized DNA fragments containing homologous flanking regions to drive the integration. This type of integration is a very rare event in *Rhizopus* that makes the construction of gene knockout mutants rather difficult (Ibrahim and Skory, 2007a). In case of *M. circinelloides* there are several reports of successful application of gene replacement to generate knockout mutants or simply to achieve stable integration. Navarro *et al.* (2001) used the method to construct *crgA* null mutants from *M. circinelloides* to examine the function of the *crgA* gene that proved to be a negative regulator of the light-inducible carotene biosynthesis. For gene knockout, they transformed a leucine auxotroph strain with a DNA fragment that contained the *leuA* gene flanked with the adjacent regions of the *crgA* gene. The *pyrG* gene could also be used for construction of knockout vectors (Quiles-Rosillo *et al.,* 2003b; Silva *et al.,* 2006). Silva *et al.* (2006) characterized the function of three white collar genes (*mcwc-1a*; *mcwc-1b* and *mcwc-1c*) that control different light-induced transduction pathways in *M. circinelloides*. For this study, they designed three vectors, each harboring the *pyrG* gene as a selectable marker, flanked by the adjacent sequences of the appropriate gene. Fragments cut from these plasmids could be used to generate knockout mutants by double crossover gene replacement. In another study, the integration event following transformation of *M. circinelloides* with linear gene replacement cassettes seemed rather variable (Larsen *et al.,* 2004). To develop an improved and constitutively expressing promoter able to drive heterologous genes in *M. circinelloides*, Larsen *et al.* (2004) constructed several expression vector that contained fragments from the promoter of the *Mucor* glyceraldehyde-3-phosphate dehydrogenase 1 gene (*gpd1*) fused with the *Aspergillus niger* glucose oxidase 1 gene (*gox1*). A leucine auxotroph strain was transformed with different linear fragments that included the corresponding promoter sequence fused with the *gox1* gene; the *leuA* gene as a selectable marker and appropriate flanking regions for targeting the integration into the *gpd2* or *crgA* loci. In majority of transformants, the introduced DNA integrated ectopically (e.g. at loci different from *gpd2* or *crgA*) and gene replacement occurred in only a few cases.

In some species, transformation with circular plasmids may also result in stable integrative transformants with a convenient frequency. Such plasmids generally include fragments that are homologous with repetitive regions of the

host genome and the transformation event obviously occurs via a single crossover (additive) integration. For example, from *Mo. alpina*, stable integrative transfo-rmants can easily be generated with circular plasmids containing an rDNA segment (Mackenzie *et al.,* 2000; Takeno *et al.,* 2004; 2005). Although majority of the resulting transformants are stable containing the introduced DNA integrated into the 18S rDNA locus, some portion of the transformants (20-40%, depending on the experimental conditions) may be unstable harboring autonomously replicating plasmids. In the case of *A. glauca*, there is an early report on stable integration of neomycin resistance that was mediated by recombination via a highly repetitive DNA element (Burmester *et al.,* 1990). This element named as *rag1* (Wöstemeyer and Burmester, 1986) corresponds to a repetitive DNA sequence that is present on all chromosomes of *A. glauca.* Transformation with *rag1* containing vectors led to transformants with integrated multiple copies of plasmids (Burmester *et al.,* 1990). Later, Schilde *et al.* (2001) included the *rag1* element in *gfp* containing expression vectors to promote the possible integration. However, integration even was not proven, moreover, original transforming plasmids harboring the *rag1* element was recovered by retransformation of *E. coli* with bulk DNA from the transformants.

As mentioned earlier, *Agrobacterium*-mediated transformation is able to efficiently transfer the foreign DNA into the host genome and the method has been adapted to a number of fungi belonging to the Zygomycetes. However, stable transformants have only been obtained from *R. oryzae* (Michielse *et al.,* 2004), while mitotic instability (e.g. complete loss of the introduced DNA) was observed in the *Rm. miehei*, *M. circinelloides* and *B. lamprospora* ATMT transformants even under selective conditions (Monfort *et al.,* 2003; Nyilasi *et al.,* 2005, 2008). The main difference between the ATMT of *R. oryzae* and that of the other species was the nature of the introduced DNA: for the transformation of *R. oryzae*, the *R. niveus pyr4* (*pyrG*) gene was used as a selectable marker, whereas different bacterial genes providing antibiotic resistance were used to transform *Rhizomucor*, *Mucor* and *Backusella*. It is suggested, that these fungi have a defense mechanism eliminating exogenous DNA via rearrangements and deletions and that stable integrative transformation can be achieved only through the introduction of endogenous or closely related DNA (Monfort *et al.,* 2003; Obraztsova *et al.,* 2004; Michielse *et al.,* 2004; Nyilasi *et al.,* 2005, 2008).

Zygomycetes fungi generally produce multinucleate spores and protoplast and form coenocytic mycelia. Thus, in the most cases, primary integrative transformants are heterokaryotic to the transferred DNA. Attainment of the homokaryotic stage frequently requires some consecutive cultivation cycles on selective medium. However, as zygomycetes have the characteristic to lose the un-integrated plasmids rapidly under non-selective conditions, one sporulation round on non-selective medium before the selective cultivation cycles may facilitate the enrichment for stable integrative transformants (Arnau *et al.,* 1991; Ibrahim and Skory, 2007a).

Expression of heterologous genes

Future exploitation of the biotechnological potential of zygomycetes (e.g. ability to produce carotenoids, polyunsaturated fatty acids, various extracellular enzymes or organic acids) urges the development of appropriate strain improvement methods. Availability of heterologous gene expression systems is crucial for various fields of biotechnological studies, such as recombinant protein production or metabolic engineering of a producer strain.

Among zygomycetes, the organism most frequently used for heterologous expression studies is *M. circinelloides*. This fungus is very amenable to molecular techniques and a number of genetic manipulation tools, such as transformation systems using autoreplicative plasmids; methods for gene replacement and *Agrobacterium*-mediated transformation, have been developed for it. Genes of related fungi can be easily expressed in *M. circinelloides*, and it has been used to test the function of several genes isolated from other species (e.g. from *Phycomyces*, *Blakeslea* and *Rhizomucor*), in which an appropriate transformation system has not been available (Iturriaga *et al.,* 1992; Ruiz-Hidalgo *et al.,* 1999; Quiles-Rosillo *et al.,* 2003a; Rodríguez-Sáiz *et al.,* 2004; Quiles-Rosillo *et al.,* 2005; Lukács *et al.,* 2009). This fungus has also been able to efficiently express genes isolated from far-related fungi (e.g. from Ascomycetes and Basidiomycetes) as well as genes from bacterial origin. For example, *M. circinelloides* was successfully used for the production of the *A. niger* glucose oxidase 1 protein (Wolff and Arnau, 2002; Larsen *et al.,* 2004); it was able to express the *Xanthophyllomyces denrorhous crtS* gene leading to increased β-cryptoxanthin and zeaxanthin production (Álvarez *et al.,* 2006) and carboxine resistance gene (*cbx*^{r}) of *Ustilago maydis* also could be used as a selectable marker for transformation of the fungus (Ortiz-Alvarado *et al.,* 2006). Heterologous expression of the β-carotene ketolase and hydroxylase genes (*crtW* and *crtZ*, respectively) of the marine bacterium, *Agrobacterium aurantiacum* was also successful: they were capable to force the production of oxygenated β-carotene derivatives, such as astaxanthin and canthaxanthin, in *M. circinelloides* (Papp *et al.,* 2006). Functional expression of the rat adenosine A1 receptor gene fused with the promoter and a segment of the *Mucor* glucoamylase gene in *M. circinelloides* (Houghton-Larsen and Pedersen, 2003) has demonstrated that the fungus maybe useful for production or analysis of even mammalian proteins.

To achieve the expression of an exogenous gene, it is usually necessary to combine it with an adequate regulatory sequence of the host. Table 3.2 presents an overview of the promoter sequences that have been used to ensure the expression of exogenous genes in zygomycetes fungi. Although a number of promoters have been identified in zygomycetes, majority of them have been used primarily to develop new transformation systems, e.g. to enable the expression of exogenous selectable markers (Wöstemeyer *et al.,* 1987; Mackenzie *et al.,* 2000) and only a few promoters has been analyzed in detail (Larsen *et al.,* 2004). One of them is the *R. niveus* 3-phosphoglycerate kinase 2 (*pgk2*) that was the first promoter analyzed using a heterologous expression system (Takaya *et al.,* 1995).

Derivatives of the promoter region harboring deletions were fused to the *Escherichia coli* β-glucuronidase gene (*uidA*), and introduced into *R. niveus* to determine the intracellular glucuronidase activities of the transformants. In this way, a short sequence was determined that was suggested to be a glucose-inducible transcriptional activator.

Table 3.2. Promoters proved to be useful to drive exogenous genes in zygomycetes fungi

Species[a]	Promoter[b]	Expressed gene	Reference
Absidia glauca	*Absidia glauca, act*	*Escherichia coli, nptII*	Wöstemeyer *et al.*, 1987
	Absidia glauca, tef	*Escherichia coli, neo*^{r}	Burmester *et al.*, 1992
	Absidia glauca, act	green fluorescent protein gene (*gfp*)	Schilde *et al.*, 2001
	Absidia glauca, tef	green fluorescent protein gene (*gfp*)	Schilde *et al.*, 2001
Backusella lamprospora	*Mucor circinelloides, gpd1*	green fluorescent protein gene (*gfp*)	Nyilasi *et al.*, 2008
	Mucor circinelloides, gpd1	*Escherichia coli, hpt*	Nyilasi *et al.*, 2008
Mortierella alpina	*Mortierella alpine, his H4.1*	*Escherichia coli, hpt*	Mackenzie *et al.*, 2000
	Mortierella alpine, his H4.1	*Zeo* (Invitrogene)	Takeno *et al.*, 2005
Mucor circinelloides	*Mucor circinelloides, gpd1*	*Aspergillus niger, cox1*	Wolff *et al.*, 2002
		Escherichia coli, kan	Appel *et al.*, 2004
		Agrobacterium aurantiacum, crtW	Papp *et al.*, 2006
		Agrobacterium aurantiacum, crtZ	Papp *et al.*, 2006
	Aspergillus nidulans, trpC	*Escherichia coli, hpt*	Nyilasi *et al.*, 2005
	Mucor circinelloides, glaM	rat adenosine A1 receptor gene	Houghton-Larsen and Pedersen, 2003
Phycomyces blakesleeanus	*Phycomyces blakesleeanus, pkpA*	*Escherichia coli, npt*	Obraztsova *et al.*, 2004
Rhizomucor miehei	*Ashbya gossypii, tef*	*Escherichia coli, aphI*	Monfort *et al.*, 2003
Rhizopus niveus	*Rhizopus niveus pgk2*	*Escherichia coli, iudA*	Takaya *et al.*, 1995
	Rhizopus oryzae, amyA	*Escherichia coli, G418*^{r}	Yanai *et al.*, 1990
Rhizopus oryzae	*Rhizopus niveus, pgk2*	*Aspergillus nidulans, amdS*	Michielse *et al.*, 2004
	Rhizopus oryzae, amyA	green fluorescent protein gene (*gfp*)	Mertens *et al.*, 2006

Rhizopus oryzae, *pdcA*	green fluorescent protein gene (*gfp*)	Mertens *et al.*, 2006
Rhizopus oryzae, *pgk1*	green fluorescent protein gene (*gfp*)	Mertens *et al.*, 2006
Rhizopus oryzae, *rFTR*	green fluorescent protein gene (*gfp*)	Ibrahim *et al.*, 2007b

[a]Name of the host organism

[b]Promoters are represented by designation of the corresponding gene

One of the best-characterized promoter is that of the *M. circinelloides gpd1* gene, which proved to be useful to drive the expression of both homologous and heterologous genes in *Mucor* (Wolff and Arnau, 2002; Larsen *et al.*, 2004; Appel *et al.*, 2004; Papp *et al.*, 2006). Transcription of *gpd1* is regulated by the carbon source: expression of the gene is significantly stronger when growing the fungus in glucose, compared with glycerol or ethanol (Wolff and Arnau, 2002). Larsen *et al.* (2004) constructed and analyzed several derivatives of the promoter. As a result of this analysis, a 361-bp derivative was found that is able to drive the expression of exogenous genes in a constitutive manner providing high expression level regardless of the carbon source used. Thus, this promoter seems to be a promising tool for constitutive heterologous gene expression in *M. circinelloides*. The functionality of the *M. circinelloides gpd1* promoter was also demonstrated in *M. rouxii* and *R. pusillus* (Appel *et al.*, 2004) arising the possibility of a broader application of this sequence.

In *R. oryzae*, promoters of glucoamylase A (*amyA*), pyruvate decarboxylase (*pdcA*) and phosphoglycerate kinase (*pgk1*) have been characterized (Takaya *et al.*, 1995; Gao and Skeen, 2002; Skory, 2003; Mertens *et al.*, 2006). Recently, Mertens *et al.* (2006) tested the applicability of these promoters to drive the expression of heterologous proteins in *R. oryzae*. They constructed expression vectors containing the promoters of *amyA*, *pdcA* and *pgk1* each fused with the green fluorescent protein gene (*gfp*) It was found that their potential to drive the expression of the *gfp* gene correlated with the choice of promoter with *pdcA* > *amyA* > *pgk1*.

Conclusions

Molecular analysis and manipulation of zygomycetes fungi have been considered especially difficult due to their unique ability to replicate the introduced DNA autonomously from the host genome. A consequence of this feature is that the majority of the transformation protocols developed for this fungal group only allow the generation of mitotically unstable transformants. Although such transformation systems can be used for some analysis (e.g. complementation analysis or expression studies), this phenomenon has hampered molecular research of zygomycetes. Functional analyses of genes, pathogenicity studies or metabolic engineering require the application of gene disruption or generation of mitotically stable transformants. During the past decade, a promising advance has been made in the development of new manipulation tools.

With the better understanding of the molecular mechanisms determining the fate of the introduced DNA, transformation techniques allowing the generation of stable integration have been successfully established in some zygomycetes model organisms, such as in *M. circinelloides* (via gene replacement using linearized fragments), *Mo. alpina* (using circular plasmids containing repetitive elements) and *R. oryzae* (by the adaptation of the ATMT method). Currently, an acceleration of molecular genetic studies on zygomycetes can be observed especially in fields related to possible biotechnological applications that inspire the further improvement of the genetic manipulation tools including heterologous expression systems.

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CHAPTER - 4

CURRENT ADVANCES IN ASPERGILLOSIS

AMPARO SOLÉ

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Introduction

Aspergillosis is a large spectrum of diseases caused by members of the genus *Aspergillus*. The principal entity is invasive aspergillosis (IA), however *Aspergillus* species can also produce a wide range of chronic, saprophytic, and allergic conditions. IA currently constitutes the most common cause of infectious pneumonic mortality in patients undergoing hematopoietic stem cell transplantation (HSCT), and is an important cause of opportunistic respiratory and disseminated infection in other immunocompromised patients as in solid organ transplantation (SOT), especially in lung transplantation (LT) patients. The other forms of aspergillosis, such as allergic bronchopulmonary aspergillosis (ABPA), allergic sinusitis, and saprophytic infection, are also causes of morbidity and seldom life-threatening. The clinical manifestation and severity of the disease depend on the immunologic state of the patient. Lowered host resistance due to factors as underlying debilitating disease, neutropenia, disruption of normal flora, and due to the use of antimicrobial agents and steroids, can predispose the patient to colonization, invasive disease, or both. *Aspergillus* spp. infection is frequently a secondary opportunistic pathogen in patients with chronic respiratory diseases, such as bronchiectasis (particularly in cystic fibrosis patients), chronic obstructive pulmonary disease (COPD) and LT.

Infectious complications after LT are frequent as a direct consequence of the aggressive immunosuppression employed in these receptors, as well as the presence of impaired mucociliary clearance, ischemic airway injury, altered alveolar macrophage phagocytic function, and direct communication of the transplanted organ with the environment.

Fungal infections (FI) are associated with a high mortality rate in LT recipients for several reasons: the difficulty of establishing an early diagnosis, the lack of effective treatment for infections by some filamentous fungi, the toxicity and interactions of some antifungal agents with immunosuppressive drugs, the scarce published experience about the use of prophylaxis with antifungal drugs in this setting, and finally the loss of grafts as the result of reducing immunosuppression to cure these infections.

FI occur in 15-35% of patients after LT and over 80% are caused by *Candida* spp. and *Aspergillus* spp., with an overall mortality rate of nearly 60% (Alexander and Tapson, 2001; Singh and Husain, 2003; Kotloff, 2004; Solé, 2005; Marik, 2006; Segal and Walsh, 2006; Iversen *et al.,* 2007). Unusual moulds as *Scedosporium* spp. are increasingly recognized as important opportunistic pathogens in LT; other moulds as Zygomycetes and species of *Fusarium* have a less relevant role in LT, but in all cases their infection is associated with a high rate of dissemination and poor outcome (Husain *et al.*, 2003; Almyroudis *et al.*, 2006). Fungal pneumonia constitutes the 14% of all causes of pneumonia in lung transplant recipients, as it is observed in a recent study of the Spanish group RESITRA (Aguilar-Guisado *et al.*, 2007). In this series *Aspergillus* spp. (8,8%) was the most frequent organism in this etiological group, but the incidence of *Aspergillus pneumonia* was lower than previously reported, probably due to the spread of universal prophylaxis.

The fungus *Aspergillus* can be found worldwide, with preferential tropism for humid soil. Numerous species have been reported, few are pathogenic and *Aspergillus fumigatus* accounts for 90% of human infections. Although *Aspergillus* spp. causes various diseases in humans, the present chapter will focus mainly on LT patients with aspergillosis. However, due to the increasing frequency of other entities, we will also discuss some aspects of invasive pulmonary aspergillosis (IPA) in COPD, and the ABPA in asthma and cystic fibrosis (CF) patients.

Epidemiology

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment.

Aspergillus is a large genus, containing over 200 species, to which humans are continuously exposed (Soubani and Chandrasekar, 2002). However, only a small number of these species have been associated with invasive infections, and over 95% of all infections are caused by no more than three species: *A. fumigatus, A. flavus* and *A. niger*. Other species typically reported in relation with aspergillosis including *A. nidulans,* this species of aspergillus (oryzae) is repeated, to eliminate one of them *A. terreus* (resistant to amphotericin B), *A. ustus* (resistant to amphotericin B, echinocandins, and azole derivatives), and *A. versicolor.* Less frequently, *A. alliaceus, A. candidus, A. chevalieri, A. clavatus,*

A. flavipes, A. glaucus, A. granulosus, A. hollandicus, A. japonicus, A. ochraceus, A. oryzae, A. restrictus, A. sydowii or *A. tamarii* have been isolated in cases of IA, mainly in immunocompromised hosts.

The physiological characteristics of *Aspergillus* spp., particularly growth rates and the production of metabolites (extrolites), often show differences that reflect phylogenetic species boundaries, and greater emphasis should be placed on extrolite profiles and growth characteristics in species descriptions. In the last years, multilocus sequence-based phylogenetic analyses have emerged as the primary tool for inferring phylogenetic species boundaries and relationships within subgenera and sections.

In 2004, *A. lentulus* was recognized as being separate from *A. fumigatus* using a multigene-approach. It has the appearance of a smaller, somewhat degenerate *A. fumigatus* with decreased conidiation, but in contrast to that species it is unable to grow at 48°C (Balajee *et al.*, 2005). This species has been reported as cause of invasive mycosis in haematopoietic stem cell recipients and has decreased *in vitro* susceptibilities to multiple antifungals, including amphotericin B (AmB) [introducing the abbreviarure] itraconazole, voriconazole, and caspofungin (Balajee *et al.*, 2004; Balajee *et al.*, 2005). More recently, *A. alliaceus* was recovered as the etiological agent of invasive pulmonary aspergillosis (IPA) and had reduced *in vitro* susceptibilities to AmB and caspofungin, which correlated with clinical failure of therapy (Balajee *et al.*, 2007). In the last years some new species have been differentiated from the original species, like *A. insuetus* or *A. calidoustus,* that differ from *A. ustus* in producing different metabolites or molecular characters and have diverse patterns of antifungal susceptibility (Houbraken *et al.*, 2007).

Identification of *Aspergillus* spp. in the laboratory

The identification of species of *Aspergillus* is not easy. For identification of aspergilli, macroscopic and microscopic characteristics of the colony are important. To aid in the identification of the medically important *Aspergillus* species, it is recommended to consult some of the excellent available guides (de Hoog *et al.*, 2000; Dupont *et al.*, 2000).

Macroscopic features. Growth rate, colour of the colony, and thermotolerance are the major macroscopic characteristics remarkable in species identification. Except for *A. nidulans* and *A. glaucus,* the growth rate is rapid to moderately rapid. *Aspergillus* colonies are velvety to powdery in texture and the surface colour may vary depending on the species. *A. fumigatus* is a thermotolerant fungus and grows well at temperatures over 40°C.

Microscopic features. *Aspergillus* spp. are filamentous organisms that reproduce by asexual spores termed "conidia". Conidia are produced in a basipetal fashion, forming chains of asexual conidia, with the youngest conidium at the base and the oldest at the tip of the chain. In the early stages of fungal growth, certain cells of the vegetative mycelium enlarge and form a heavy wall.

These particular cells, the foot cells, will form a branch at a right angle to the parent cells. The branch formed, which develops into a conidiophore, terminates in a swollen head known as a vesicle. The length of conidiophore and the nature of its wall are considered important characteristics of the species. The vesicles vary in size and shape depending on the species. Certain areas of the vesicle surface become fertile and give rise to a layer of conidium producing cells: the phiallides. In some species these phiallides cover the entire surface of the vesicle, whereas in others the may cover the upper half or three quarters of the surface. Furthermore, they may vary in color in different species. The cylindric body of the phiallides narrows at the apex to form a conidium-producing tube. Conidia are usually globose with a rough surface and variable sizes. The length of conidial chains, density of packing and orientation around the vesicle vary from species to species and are considered part of the distinctive characteristics to identification.

Risk factors of Aspergillosis

The incidence of IA in non-neutropenic patients is probably underestimated, and it is due to the poor sensitivity of clinical and radiological investigations and diagnostic tests. Some reports about the incidence of IA in patients who were hospitalized for underlying diseases, such as COPD, asthma, rheumatoid polyarthritis, giant cell arteritis, and vasculitis have been recently published (Meersseman *et al.*, 2004; Cornillet *et al.*, 2006). Receiving high-doses of corticosteroids or continuous corticosteroid therapy were the only identified risk factors for 22% of patients. However, low steroid doses (15 mg of prednisone per day or equivalent) were sufficient for IA to develop when the medication was administered continuously. In these moderately immunosuppressed patients, *Aspergillus* colonization may contribute to the onset of IA, particularly in patients with chronic pulmonary disease. Besides, corticosteroids, intercurrent bacterial, cytomegalovirus, and *P. jiroveci* pneumonia are frequent, particularly among non-neutropenic patients, underlining that the presence of *Aspergillus* in respiratory samples (in addition to another infectious agent) should not be systematically considered to represent simple colonization (Garnacho-Montero *et al.*, 2005). In summary, a wide range of risk factors must be taken into account, including continuous corticosteroid therapy (even at low doses), co-infections and *Aspergillus* colonization of the respiratory tract.

Environmental factors may also increase the risk of infection with *Aspergillus* and other filamentous moulds in low-risk patients. Such factors include demolition and construction in the vicinity of the hospital, and contamination or malfunction of the air-conditioning system near patient care areas (Bouza *et al.*, 2002).

It is also possible that hospital water supplies may be a potential source of *Aspergillus* spp. (Anaissie *et al.*, 2002). In fact, an invasive mycosis in an otherwise low-risk patient should trigger an investigation into environmental factors and the use of widespread prophylaxis until the problem is solved.

On the other hand, in SOT, invasive mycoses are concentrated in specific subpopulations of transplant recipients. A complicated postoperative course, repeated bacterial infections, concomitant cytomegalovirus (CMV) infection, and renal replacement therapy seem to significantly increase the risk for early (within the first 3 months after transplantation) IA infections. Risk factors for late onset fungal infections include advanced age, renal failure, and an increased immunosuppressive state due to chronic rejection (Cahill *et al.*, 1997; Gordon and Avery, 2001; Gavalda *et al.*, 2005; Singh and Paterson, 2005). Patients receiving LT seem to be particularly susceptible to infection by *Aspergillus* spp. Risk factors for *Aspergillus* infection include colonization prior or post transplant, single LT, CMV infection, chronic rejection, and the type of antifungal prophylaxis. Regarding *Aspergillus* colonization, it has been proven that prior colonization does not imply the development of invasive pulmonary aspergillosis (IPA). In fact, in our experience, patients suffering from CF colonized by *Aspergillus* before transplantation do not have a higher incidence of pulmonary or disseminated disease after LT. However, they are at increased risk of anastomotic infections in the early post-transplantation period (Solé *et al.*, 2005). Nevertheless, *Aspergillus* colonization before LT has been associated with a higher incidence of invasive infection. Studies report that 6% (range 3% to 20%) of patients with colonization before transplantation develop invasive disease (Singh and Husain, 2003; Gavalda *et al.*, 2005; Singh and Paterson, 2005).

It has been demonstrated that LT patients colonized with *Aspergillus* in the first 6 months after transplantation were 11 times more likely to develop invasive disease than were non-colonized patients (Cahill *et al.*, 1997). CMV disease is another risk factor for IA infection in LT recipients. In fact, a large number of the LT with IA have concurrent CMV disease (Alexander and Tapson, 2001).

Additionally, some studies have shown a direct relation between the use of some immunosuppressive drugs and invasive mycoses (cryptococcosis, aspergillosis), mainly when they are used as antirejection (Silveira *et al.*, 2007) or post transplant lymphoproliferative disorder (PTLD) therapy (Martin *et al.*, 2006). In our series we have not found any association with immunosuppressive therapy, neither with the use of high corticosteroid doses nor with the use of tacrolimus, being chronic rejection the only significant association which we have found (Solé *et al.*, 2005).

Diagnosis

The diagnosis of invasive mycoses in immunosuppressed patients poses significant clinical challenges. In fact, neither radiological findings (patchy infiltrates or consolidation) nor respiratory samples have a high specificity. Symptoms of invasive mycoses are non-specific, and initially about a 30% of cases are asymptomatic. Besides, *Aspergillus* is cultured from sputum in only 8-34%, and from bronchoalveolar lavage (BAL) fluid up to 62% of patients with invasive disease. Moreover, post LT airway colonization arises up to 55% (false

positive). In fact, data revisions about aspergillosis in lung transplant have demonstrated the paucity sensibility of an airway culture positive for *Aspergillus* in evidencing IA (Singh and Husain, 2003; Segal and Walsh, 2006).

Laboratory diagnosis

Microscope and Culture

The diagnosis of aspergillosis could be improved with the microscopic observation of sputum, since septate mycelium with dichotomous branching is usually seen in this clinical sample. Microscopic examination using a wet mount or calcofluor white stain of BAL, biopsies or sinus washings is often rewarding in patients with suspected invasive aspergillosis. However, the definitive diagnosis of IA requires the isolation of the etiologic agent in culture, but since *Aspergillus* spp. are commonly found in the air, their isolation in the upper respiratory tract samples must be interpreted with caution. *Aspergillus* isolation from sputum is more suggestive of IA if multiple colonies are recovered in a plate or if the same fungus is recovered on several occasions. In contrast, isolation of an *Aspergillus* from a BAL is often an indicative of infection but, unfortunately, it is positive in less of 60% of cases (Nalesnik *et al.*, 1980; Delvenne *et al.*, 1993; McWhinney *et al.*, 1993).

Immunodiagnostic

Given that diagnosis of IA can be challenging, recent efforts have focused on non-culture-based methods to establish a rapid diagnosis. Immunological techniques have been described years ago for diagnosis of aspergillosis. Procedures based on antibody detection have been successful in aspergilloma and allergic aspergillosis, and those used for detection of fungal antigen have great potential for the diagnosis of invasive aspergillosis.

Currently, detection of specific precipitating antibodies to *Aspergillus* spp. are used for the diagnosis of ABPA, and the presence of one or more weak precipitins bands is one of the diagnostic criteria accepted for diagnosis of this entity (70-100% of patients with ABPA are positive for IgG-precipitating antibodies against *Aspergillus* spp.) (Nelson *et al.*, 1979). But antibody production in the immunocompromised patient with IA is habitually difficult to detect. Therefore, methods for diagnosing this infection that would rely on the measurement of fungal cell components and thus be independent of the host's response have been required. During infection, *Aspergillus* galactomannan (GM), a polysaccharide cell wall component, is actively released and could be detected in serum; however, GM is quickly cleared by Kupffer's cells and its levels in serum are irregular. The detection of GM by sandwich-enzyme immunoassay (EIA) has been approved by the U.S. Food and Drug Administration (FDA) for use in HSCT recipients, but there is few data in SOT (Zaas and Alexander, 2005; Pfeiffer *et al.*, 2006). In a study of 70 lung transplant recipients, the sensitivity of

serum GM for the diagnosis of IA was low (Husain *et al.*, 2004). The test detected only 30% of the cases of IA and none of the cases of tracheobronchitis (cut-off value of ≥ 0.66). In a meta-analysis its sensitivity and specificity for SOT was 0.41 (range 21-64) and 0.85 (range 80-89), respectively (Pfeiffer *et al.*, 2006). Thus, the test has demonstrated excellent specificity, but a low sensitivity for the diagnosis of aspergillosis in this patient population.

Transplanted CF or COPD patients may transiently have a GM positive test in the early post-transplant period. Given that, alternate specimens such as BAL may prove to be advantageous in this population. The presence of GM in the BAL fluid is therefore likely to be a better diagnostic indicator for hyphal growth than routine mycological culture. The unique study about the role of GM antigen in BAL for the diagnosis of IA has been assessed recently in LT recipients (Husain *et al.*, 2007). At the index cut-off value of ≥0.5, the sensitivity was 60%; specificity was 95%. Increasing the index cut-off value to ≥1.0 yielded a sensitivity of 60%, a specificity of 98%. Hence, an index ≥1.0 in the BAL fluid in a LT recipient with a compatible clinical illness may be considered as suggestive of IA. However, patients with IA receiving antifungal prophylaxis (voriconazole, itraconazole) could have false-negative results. False-positive GM tests have also been reported in patients receiving piperacillin-tazobactam in serum and BAL (Husain *et al.*, 2007). The value of GM in BAL fluid in the diagnosis of IPA among solid-organ transplant recipients has been recently reported (Clancy *et al.*, 2007). Sensitivity, specificity, and positive and negative predictive values for BAL GM being tested at a cutoff of ≥ 1.0 were 100%, 90.8%, 41.7%, and 100%, respectively. The sensitivity of BAL GM testing was better than that of conventional tests such as serum GM or BAL cytology and culture. Moreover, a positive BAL GM test diagnosed IPA several days to 4 weeks before other methods for three patients. Among LT recipients, 41.7% accounted for false-positive results, reflecting frequent colonization of airways among this population. Excluding lung transplants, the specificity and positive predictive value for other solid-organ transplants increased to 92.9% and 62.5%, respectively (cutoff ≥ 1.0). Therefore, BAL GM testing facilitated more-rapid diagnoses of IPA and the institution of antifungal therapy among non-lung solid-organ transplant recipients, and helped to rule out IPA. However, multicenter studies are needed to establish the diagnostic value of GM in IA in SOT recipients.

Other non-culture based techniques

The cell wall of *Aspergillus* hyphae and other pathogenic fungi consist of mannans and glucans. (1→3)-β-D-glucan is a cell wall polysaccharide found in fungi, except zygomycetes and cryptococci, that may be a marker of invasive fungal infection (IFI) with the most common pathogens such as *Aspergillus* and *Candida*. Sensitivities reported in the literature of the (1→3)-β-D-glucan assay (Glucatell®) are variable, and controlled clinical trials regarding its use in patients at risk for IA in LT patients are unavailable. But there are some studies that

include a few SOT (majority cancer and allogenic HSCT) which show (cut-off ≥ 80 pg/ml) a sensitivity of 0.71 and specificity of 0.86 for invasive mycoses diagnosis (Koo *et al.*, 2006). Although a positive result does not indicate the specific cause of IFI, this approach is encouraging and warrants more extensive investigation in selected patient populations. Hence, detection of (1→3)-β-D-glucan may be useful in diagnosis of IFI but it must be join to other identity fungal tools.

Finally, the appearance of new molecular techniques lays a new way in the diagnosis of the fungal infection: the nucleic acid detection. The use of polymerase chain reaction (PCR) to detect invasive fungal pathogens has been reported, but false positive results have limited its clinical use. The ubiquitous nature of some fungi in patient samples and in the air has resulted in false-positive results. Thus, further evaluation of this molecular assay is needed. A recent meta-analysis to obtain an overview of the diagnostic accuracy of PCR techniques (real-time PCR, PCR-ELISA and nested PCR) for the diagnosis of IA has been performed in immunosuppressed patients (mostly with haematological disorders). The analysis concluded that PCR had a sensitivity and specificity of 0.70 and 0.90, respectively. But, despite these good results, the paucity of standardized methods decreases their use because of the difficulty to compare the obtained results (Cruciani *et al.*, 2006). The fundamental disadvantages of these techniques are high cost, necessity of specialized personnel, lack of standardized commercial tests, and lack of studies that demonstrate what type of sample and procedure is indicated (Gadea *et al.*, 2007). In summary, studies of the sensitivity and specificity of PCR assays for IA in SOT patients are required to establish their diagnostic value.

Radiology

With regard to radiology IPA may appear as single or multiple nodular opacities, cavities, or alveolar consolidation (Fig. 4.1, 4.2, 4.3). In fact, plain chest x-rays and computed tomography (CT) scan are insensitive and non specific in lung transplant patients. The halo sign, considered a highly characteristic radiographic early feature in neutropenic patients, is infrequently encountered and considerably less specific in SOT populations (Vilchez *et al.*, 2002; Brodoefel *et al.*, 2006; Greene *et al.*, 2007). Recently, in a retrospective analysis of a large cohort of SOT patients (Copp *et al.*, 2006) it has been demonstrated that several radiological findings and patient characteristics are independently associated with a specific etiology of pulmonary nodules (PNs). Therefore, radiological feature of consolidation was strongly associated with infectious etiology regardless of the organ transplanted, and that Epstein-Barr virus (EBV) seronegativity and LT (compared with other organ transplant type) were strongly associated with PTLD. In addition, PNs found early in the post-transplant period (less than 90 days) were much more likely to be due to *Aspergillus* than those that were diagnosed after 90 days. Besides, radiographic features (nodule characteristics or size, distribution) were poorly correlated with

the ultimate PNs etiology. These findings have the potential for assisting in the selection of empiric therapy in the SOT recipient with PNs, at least until the results of definitive diagnostic studies become available.

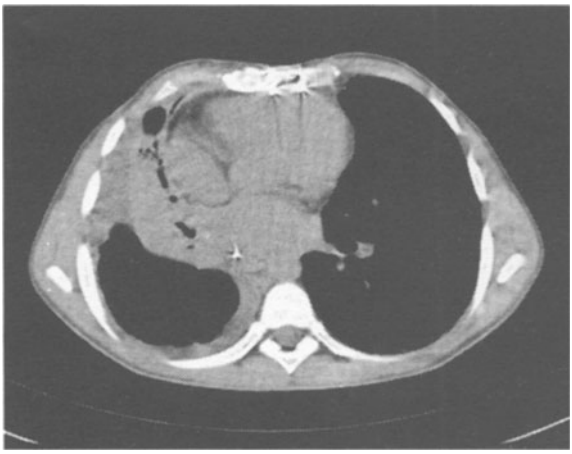

Fig. 4.1: Pattern of alveolar consolidation due to invasive pulmonary aspergillosis. Thoracic CT-scan.

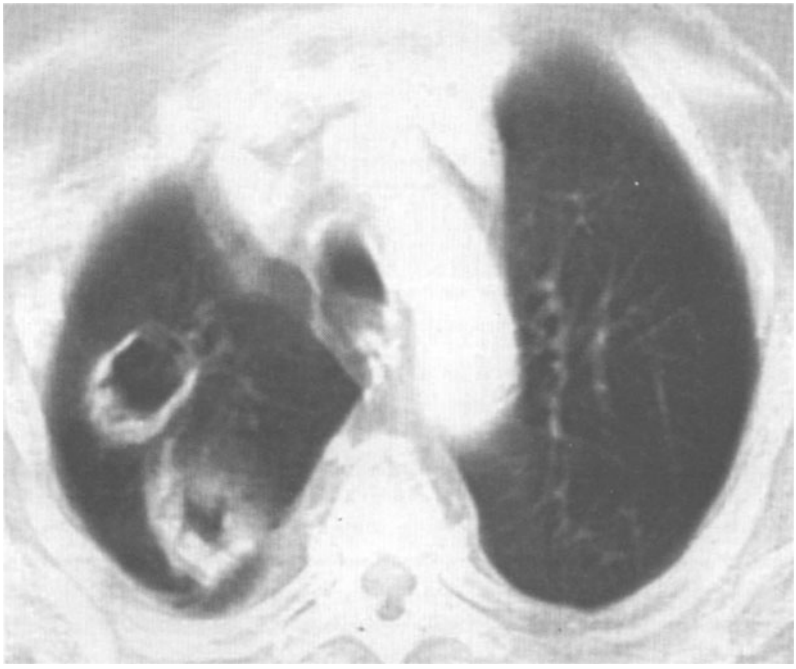

Fig. 4.2: Pattern of nodular cavities due to invasive pulmonary aspergillosis. Thoracic CT-scan.

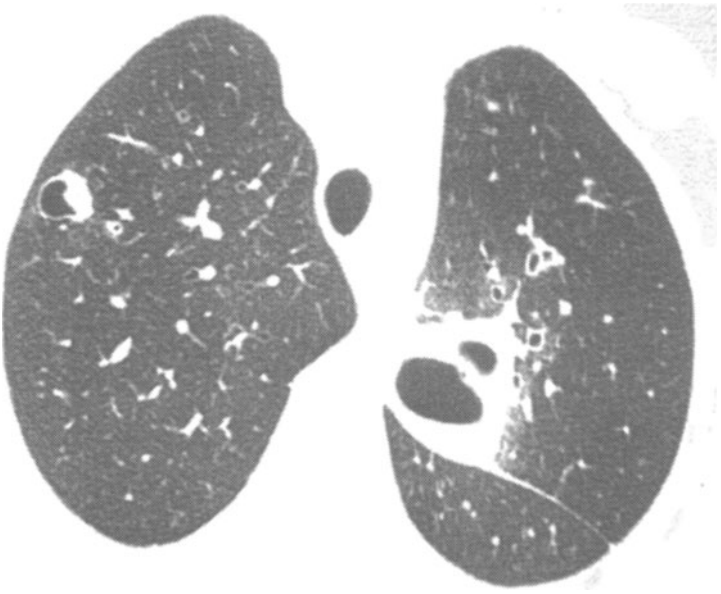

Fig. 4.3: Pattern of "air crescent" due to invasive pulmonary aspergillosis (right side). Thoracic high resolution computed tomography scan (HRCT-scan).

Other method as positron-emission tomography with 18-fluoro-2-deoxyglucose (^{18}FDG PET) for the diagnosis of invasive mycoses has been investigated recently in immunocompromised patients with proven IFI. ^{18}FDG PET revealed an increased uptake which corresponded to infected areas visualized by conventional radiographic tools, and it even disclosed small lesions unapparent on the CT scan. This study concluded that ^{18}FDG PET is useful for the diagnosis and staging of IFI; but whether or not ^{18}FDG PET might be useful for assessing duration of IFI therapy should now be assessed on a larger scale basis (Hot *et al.*, 2006).

Aspergillus spp. infection in LT recipients

A*spergillus* infections remain among the most significant opportunistic infections after LT. *A. fumigatus*, the most pathogenic species, produces the majority of infections; however, *A. flavus, terreus* and *niger*, have been increasingly reported in IFI. Data from the compilation and synthesis of existing studies give a variable incidence of *Aspergillus* infection of 6% in the published series (ranged from 2.2% to 30%). These wide ranges translate the differences in definition criteria for *Aspergillus* infections, immunosuppressive therapy and antifungal prophylaxis existing in each LT programme. It is known that infection by *Aspergillus* may manifest in LT recipients in three different forms: (i) colonization, (ii) tracheobronchitis/anastomotic infections, or (iii) invasive pulmonary/disseminated aspergillosis (Nicod *et al.*, 2001). Although cases of ABPA have been reported, this is a rare entity that only occurs in transplant

patients with CF (Helmi *et al.*, 2003). To avoid confusion about *Aspergillus* infection, the following definitions are used in this review:

1. *Aspergillus* airway colonization: patients with *Aspergillus* cultured from the airway specimens in the absence of IA or tracheobronchitis.

2. Tracheobronchial aspergillosis or anastomotic infections: isolation of *Aspergillus* in culture with histopathological evidence of tissue invasion or necrosis, ulceration or pseudomembranes on bronchoscopy.

3. Invasive pulmonary aspergillosis: IFI of the lung caused by *Aspergillus* spp., with clinical, radiological and or histological findings of pulmonary tissue invasion by *Aspergillus*, together with isolation of the fungus from respiratory samples. IPA is considered to be disseminated when the infection is documented histopathologically at two or more non-contiguous organ sites.

Aspergillus colonization usually occurs in up to 30% of patients during the first six months after transplantation and it is considered a risk factor to develop IPA. In a single-center study the isolation of *Aspergillus* spp. from respiratory samples was reported to precede acute rejection, and could be an early marker of graft dysfunction or airway inflammation (Solé *et al.*, 2005).

Regarding airways lesions, three different patterns of *Aspergillus* tracheobronchitis have been described: (i) Obstructive bronchial aspergillosis, a condition in which thick mucous plugs filled with *Aspergillus* are found in the airways, with little mucosal inflammation or invasion; (ii) Ulcerative tracheobronchitis, in which there is focal fungal invasion of the tracheobronchial mucosa and/or cartilage; (iii) and finally pseudomembranous tracheobronchitis, which is characterized by extensive inflammation and invasion of the tracheobronchial tree with a pseudomembrane composed of necrotic debris and *Aspergillus* hyphae overlying the mucosa. In LT patients, *Aspergillus* has a propensity for invading bronchial anastomoses, which leads to endobronchial complications such as excessive granulation, bronchial stenosis, dehiscence, necrosis and bronchoarterial fistula in up to 18% of LT patients (Fig. 4.1). Affected patients tend to present prominent dyspnoea, cough, and wheezing; they occasionally expectorate intraluminal mucous plugs. The chest radiograph may be normal or reveal areas of atelectasis. Isolated tracheobronchitis and bronchial anastomotic infections are different entities than *Aspergillus* pneumonia. In one study, although the early mortality of patients with bronchial anastomotic *Aspergillus* infections did not differ significantly from patients without these infections, their long-term survival was reduced (Hadjiliadis *et al.*, 2000). In our experience, anastomotic infection was associated with a significant reduction of survival rate in single LT (4/5 cases, 80%). In fact, close monitoring and pre-emptive antifungal therapy is recommended for patients with bronchial airway mechanical abnormalities and persistent *Aspergillus* colonization, because of their progression to invasive pulmonary forms.

Pulmonary involvement is the most common presentation of IA in LT patients. The major manifestation is fever. Fever is unresponsive to broad

spectrum antibiotics and often is also unresponsive to amphotericin B, which is empirically prescribed when patients remain persistently febrile. Chest pain, cough, and haemoptysis may also appear. The combination of pleuritic chest pain, dyspnoea, and haemoptysis may suggest the presence of vascular invasion due to fungal infection. However, this typical presentation is not frequent in non-neutropenic patients. The chest X-ray may be normal or reveal nodular lesions, patchy infiltrates, or cavitary lesions. CT imaging may be particularly useful in the course of IPA, when chest X-ray results are normal.

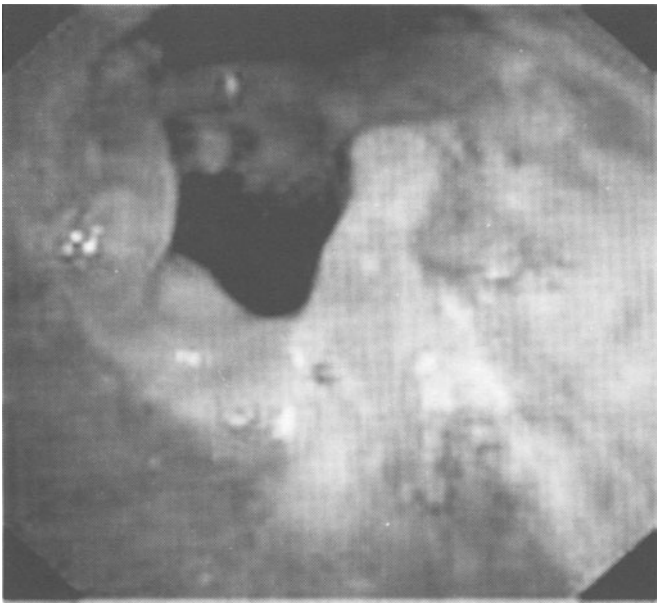

Fig. 4.4: Endobronchial bronchoscope view. Anastomotic bronchial infection due to *Aspergillus fumigatus*.

The incidence of IPA forms in LT is around 5 to 10%, and although it depends on several factors, in general they appear in severely immunosuppressed patients. Infection may disseminate beyond the respiratory tract in patients who are seriously immunocompromised (Fig. 4.5). Common clinical manifestations include fever, signs of sepsis, and infection of any organ. However, these recipients may not manifest fever despite the invasive infection. Infection of any organ can virtually occur, but most commonly, kidney, liver, spleen, and central nervous system are involved. Pathology and clinical manifestations in these areas also reflect the vascular tropism of the fungus. Skin lesions are less common.

Time of onset differs for various types of *Aspergillus* infections. IPA or disseminated aspergillosis occurred significantly later than tracheobronchitis. Usually, of the AI occurring within 3 months of transplantation, 75% are tracheobronchitis or bronchial anastomotic infections, 18% are invasive pulmonary infections and 7% are disseminated invasive infections (Singh and

Husain, 2003). Historically, the vast majority of AI in LT occurred within 90 days of transplantation (Mehrad *et al.*, 2001). In a previous report (Singh and Husain, 2003), AI occurred in 72% of cases within 6 months of LT, and only 12% were documented after 12 months of transplantation. Nowadays, characteristics of transplant recipients developing IA and immunosuppressive regimens are continuing to evolve. Thus, nearly one-half of the AI in transplant recipients in the current era are late-occurring (Singh *et al.*, 2006b; San Juan *et al.*, 2007). In fact, in our experience invasive forms were late onset (16/19) and the main risk factor was chronic rejection (Solé *et al.*, 2005). These data have relevant implications for prophylactic strategies and guiding clinical management of transplant recipients which presented pulmonary infiltrates. In our large single-centre cohort of LT, the time of onset was strongly related to the clinical form of aspergillosis. All tracheobronchitis or bronchial anastomotic infections occurred within 3 months of transplantation. In contrast, invasive or disseminated aspergillosis was significantly later (33.7 ± 19.6 months post-transplantation). All the early invasive pulmonary forms (25%) were simultaneous with tracheobronchial *Aspergillus* infection.

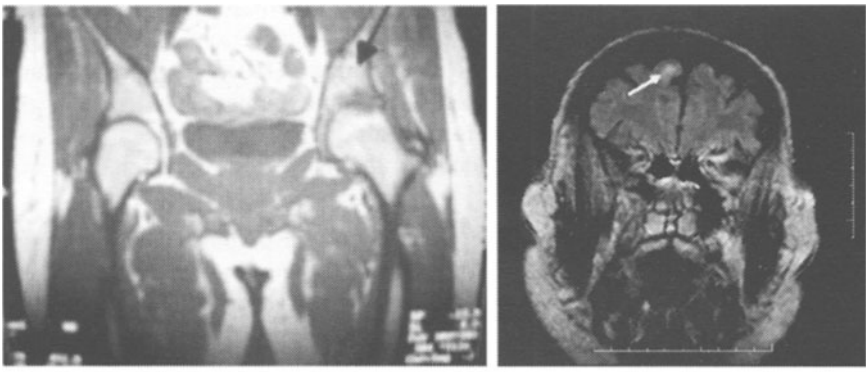

Fig. 4.5: Disseminated aspergillosis. A: Pelvic infection at hip joint (pelvic magnetic resonance imaging). B: Cerebral abscess (cerebral magnetic resonance imaging)

With respect to mortality, IA accounts for 9% of deaths in LT recipients (Husain *et al.*, 2006a). Overall mortality in LT recipients with AI is between 52% - 80% and varied significantly with the site of infection. While mortality rate is around 23% for patients with tracheobronchial or bronchial anastomotic infections, it is up to 82% for patients with IPA. Patients with late onset AI had significantly higher mortality than those with early onset infections. However, when only patients with IA were analyzed, the mortality rate did not differ for those with late versus early-onset AI (Singh and Husain, 2003). In a large series in LT, AI was associated with a reduction in the 5-year survival rate, especially in single LT recipients with bronchial anastomotic infection, and in those with late onset infections and chronic rejection.

With regard to the treatment, voriconazole, an extended spectrum highly lipophilic triazole, is actually the first choice for initial therapy of IA in LT patients and other immunosuppressed hosts. Other potentially effective therapies include lipid formulation of AmB and echinocandins. Combination therapy using a triazole and an echinocandin has been evaluated in SOT with a significant reduction in mortality in those patients with renal failure and infected by *A. fumigatus* (Singh *et al.*, 2006a).

Aspergillosis and Immune Reconstitution Inflammatory Syndrome

All too often in the treatment of fungal infections, therapeutic failure is identified as our inability to kill the invading yeasts or moulds. Our therapeutic focus has been to provide as rapid and as effective as possible an immune reconstitution in immunosuppressed patients with refractory fungal infections. However, although host immunity is crucial in the eradication of infection, immunological recovery can also be detrimental and may contribute to worsen the disease (Singh and Perfect, 2007). For instance, soon after the advent of potent antiretroviral therapy, successful immune restoration in HIV-infected patients became associated with an exuberant inflammatory response and worsening clinical manifestations of opportunistic infections. This entity, known as immune reconstitution inflammatory syndrome (IRIS), is also seen in other immunocompromised hosts, and even in immunocompetent individuals (Cheng *et al.*, 2001). IRIS is best characterised as a collection of localised and systemic inflammatory reactions of varying degrees that have both beneficial and noxious features during an invasive mycosis.

However, the concept of IRIS and its precise diagnosis in the context of opportunistic mycoses remains poorly characterised for health-care providers. Occurrence of IRIS is almost always construed as failure of therapy or a relapse caused by inability to eliminate the fungus, often leading to unwarranted or inappropriate changes in specific antifungal treatment (Jenny-Avital and Abadi, 2002). Accordingly, its management is driven by sophisticated image studies and non-specific clinical signs and symptoms of inflammation rather than precise gauging of immunological recovery. Development of IRIS also contributes to increased health-care costs and resource use.

IRIS has been observed in 5% of the solid-organ transplant recipients, a median of 5.5 weeks after the start of antifungal therapy. Transplant recipients with IRIS are more likely to have received a potent immunosuppressive regimen than those without IRIS. After reduction of immunosuppressive therapy, a relative increase in Th1 response may occur in patients receiving more potent immunosuppression and therefore a higher risk for the occurrence of IRIS. IRIS illustrates the complex host–parasite interactions in the evolution of opportunistic mycoses. IRIS in fungal infections has existed for years. However, the use in current medical practice of potent immunomodulators with their ability to rapidly alter immunological status has heightened its relevance.

Cytokine-secreting effector T cells play a major part in mediating immune responses to self and foreign antigens. Th1 cells, characterised by the production of interferon-γ, elicit proinflammatory responses. Th2 cells produce antiinflammatory and immunosuppressive cytokines (eg., interleukin 10). The cells that secrete transforming growth factor β are termed Th3 cells. These, in concert with Th2 cells, inhibit the development and function of Th1 cells. A normally functioning immune system is the result of fine balance between Th1 and Th2 or Th3 cells. An imbalance characterised by an inadequate or excessive expression of either response can be detrimental to the infected host. Th1 cytokines are also the primary mediators of allograft rejection and are the main targets of immunosuppressive agents in transplant recipients. Corticosteroids, although less potent inhibitors of Th1 than calcineurin inhibitors, are also associated with a decrease in inflammatory responses. Thus, iatrogenic immunosuppression in transplant recipients is associated with a dominant anti-inflammatory response. Reduction or withdrawal of these potent immunosuppressive agents can rapidly lead to a shift towards a proinflammatory phenotype, particularly if an invading pathogen is established in host tissue during immune suppression.

In "the world of mycoses", although IRIS has been reported in HIV patients with *Histoplasma capsulatum* (Breton *et al.*, 2006), *Pneumocystis jiroveci* (Koval *et al.*, 2002), and *Aspergillus* infections (Sambatakou and Denning, 2005), the syndrome is best characterised, in context of *C. neoformans* infection (Broom *et al.*, 2006). An IRIS–like illness associated with *Cryptococcus neoformans* infection in SOT recipients has been best characterized and recently described by Singh *et al.* (2005). The proposed basis of this phenomenon is reversal of a predominantly Th2 response at the onset of infection to a Th1 proinflammatory response as result of a reduction or cessation of immuno-suppressive therapy. This study demonstrated that an IRIS-like entity occurs in organ transplant recipients with *C. neoformans* infection. In the future, it is possible that an IRIS-like process can be described in more high frequency in SOT patients with other IFI, as aspergillosis.

The optimum management of IRIS is dependent on the awareness of its existence by health-care providers. Recognition that IRIS is a manifestation of a poorly controlled inflammatory response rather than direct treatment failure of antifungal agents to eradicate or kill the fungus- is crucial for avoiding unnecessary modifications in therapy. Currently, there are no readily available markers that can reliably establish the diagnosis of IRIS. Rationale strategies can also be applied to the management of immunosuppression in transplant recipients with these infections. Withdrawal of immunosuppression in transplant recipients with opportunistic infections is a common practice and is intuitively logical. However, concurrent withdrawal of immunosuppression and initiation of antifungal therapy has been shown to predispose not only to IRIS, but also to allograft loss (Singh *et al.*, 2005). Thus, it is plausible that spacing or separating the reduction in post-transplant immunosuppression and initiation of antifungal

therapy is a more prudent approach to the management of transplant recipients with cryptococcosis or aspergillosis. There is no proven therapy for IRIS. However, empirical treatment of symptomatic IRIS in case reports or case series has been attempted using anti-inflammatory agents (Cheng *et al.*, 2000; García *et al.*, 2005).

On the basis of preliminary data and until definitive studies are done, the use of tapering doses of corticosteroids over 6-8 weeks is a reasonable option. The role for immunomodulatory therapies targeted towards neutralization of suppressive cytokines, enhancement of Th1 responses with interferon-γ, and transfer of adoptive cellular immunotherapy has also been supported (Pappas *et al.*, 2004).

In summary, IRIS exists and we must take it into account. Characterization of clinical variables predisposing to IFI-associated IRIS, and identification of diagnostic markers merit future studies. The treatment of IRIS remains empirical, with little precision for agent, dose, or duration. Immunomodulatory therapies are potentially promising and necessary as adjuncts in the management of fungal infections, but a balance in the modulation of the immune response will be essential.

Aspergillus and Chronic Obstructive Pulmonary Disease

Aspergillus spp. cultured in specimens from the airways of COPD patients is frequently considered as a contaminant. However, growing evidence suggests that severe COPD patients are at higher risk of developing IPA, although IPA incidence in this population is poorly documented. Some data report that COPD is the underlying disease in 1% of patients with IPA. There are some topics still answered in COPD, as in LT receptors, so future research is needed to better identify *Aspergillus* infection in COPD patients as: Incidence of IPA versus *Aspergillus* colonization in COPD population, role of steroids in predisposing to IPA, diagnosis, treatment, and possible prevention.

An early diagnosis therefore seems crucial to improve outcome. In fact, IPA should be considered in COPD, in particular in severe (Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV) steroid-dependent patients with antibiotic-resistant pneumonia and exacerbated dyspnoea with or without *Aspergillus* airway colonization. The term tracheobronchitis is used when *Aspergillus* organisms invade the tracheobronchial tree. This has several degrees and ranges: from only inflammation of the mucosa without invasion and with secretions containing *Aspergillus* spp., to more invasive bronchial aspergillosis, such as pseudomembranous tracheobronchial aspergillosis and ulcerative tracheobronchial aspergillosis (Hope *et al.*, 2005).

Epidemiology

Assessing the incidence of IPA in this population is not easy due to the lack of a consistent case definition and the absence of infection surveillance measures. Moreover, colonization by *Aspergillus* spp. is often difficult to distinguish from

IPA, particularly at an early stage. However, there is growing evidence to suggest that COPD patients are at risk of IPA (Rodrigues *et al.*, 1992; Patterson *et al.*, 2000). Steroids are believed to play a role in the emergence of IA, and some authors have investigated the correlation between the daily dose of corticosteroids and the probability of developing IA. Although precise dosages or durations of corticotherapy cannot be extrapolated from the literature, data support the fact that COPD patients are at risk for IPA when they have received high doses of corticosteroids or when steroids have been administered for a long time (Muquim *et al.*, 2005). Recently, some reports have suggested that high doses of inhaled corticosteroids may also be a risk factor for IPA (Barouky *et al.*, 2003). Moreover, viral infection, such as influenza or cytomegalovirus may precede IPA, suggesting a role in causation (Rello *et al.*, 1998; Bulpa *et al.*, 2001; Ader *et al.*, 2005).

Clinical features of IPA in COPD

The main clinical sign in COPD patients with IPA is a non-specific antibiotic-resistant pneumonia associated with exacerbated dyspnoea. A significant bronchospastic component is documented in 79% of patients. As described in the literature (Bulpa *et al.*, 2007), fever is present in only 38.5% of patients. Chest pain and haemoptysis were rarely described in COPD patients. Therefore, in severe steroid-dependent COPD patients, the presence of a dyspnoea exacerbation and poor clinical status, despite the use of broad-spectrum antibiotics and high doses of steroids, is highly suggestive of IPA, especially when a recent pulmonary infiltrate appears on chest radiograph and/or when *Aspergillus* species is retrieved in the sputum. In the case of a high-probability, but not confirmed, IPA, a diagnostic procedure must be performed to confirm the presence of the disease, and treatment should be strongly considered.

Diagnostic procedures

Respiratory samples

Although COPD patients may be colonised with *Aspergillus* spp, its presence in the sputum must not be trivialised, especially in cases of antibiotic-resistant pneumonia. Direct examination of the sputum for *Aspergillus* spp. can be rapidly performed, but its positivity reaches 48% in the case of IPA (Kahn *et al.*, 1986; Segal and Walsh, 2006). It remains unclear why some patients are only colonised by *Aspergillus* while others develop IPA without positive sputum. Colonization may correspond to a temporary passage of *Aspergillus* in the tracheobronchial tree, a long-term benign carriage, or the sign preceding invasive disease (as the incubation period before IPA is unknown). In contrast, the absence of positive sputum culture during IPA may be due to the low sensitivity of the diagnostic method, to the presence of only few *Aspergillus* and mostly in their non germinated form in the lesions, or to the tendency of *Aspergillus* to invade the vessels and to induce lung infarction (Latge, 1999). When no sputum is available, material for culture should be obtained by bronchoscope

(endotracheal aspirate, BAL, or bronchial washings or brushings). The yield of cultures has ranged 46–77% (Segal and Walsh, 2006). BAL-positive microscopy for *Aspergillus* is highly suspicious of active disease, although it is unable to definitively differentiate between colonization and infection. While definitive confirmation of infection requires biopsy, immediate initiation of antifungals should be considered. Unfortunately, despite its usefulness, performance of flexible bronchoscopy in COPD patients is sometimes difficult due to their poor lung status and function. Moreover, due to its poor yield, transbronchial lung biopsies must be performed only when the infection does not respond to adequate antifungal therapy.

Serology

The current GM antigen test (ELISA test, Platelia Aspergillus; Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) has been demonstrated to be useful in haematological malignancies, in which it demonstrated a benefit as the diagnosis could be achieved before the appearance of clinical signs. However, there are only few reports in COPD. Recently, antigenaemia was tested in critically ill COPD patients with suspected IPA. Its sensitivity was 48%. In view of these scarce results no firm conclusions could be drawn (Meersseman *et al.*, 2004). Besides, the Platelia® test has several limitations related to false-positive results. In fact, false positives have been reported in patients receiving piperacillin/tazobactam or amoxicillin/clavulanate (Viscoli *et al.*, 2004) and cross-reactions with other fungi. Finally, it is unknown whether the results observed in haematological malignancies could be extrapolated to patients with other underlying diseases, such as COPD. In addition to serological tests, other blood analyses are under development, including PCR tests and dosage of plasma concentration of the b-D-glucan, but there are no results in COPD patients.

Radiology

Chest radiographs may be normal in the early stages of IPA. When present, multiple radiological signs have been described as being associated with IPA, such as consolidation nodules, the ''halo sign'', and the ''air-crescent sign''. The latter two signs are almost pathognomonic of IPA in neutropenic haematological patients. Although these signs are well described during neutropenia, it seems they are less frequently observed in COPD and solid organ-transplant patients (White, 2005; Segal and Walsh, 2006), where nonspecific consolidations are more frequently seen than the specific features mentioned.

Lung biopsy

Only histology can confirm IPA, and although transbronchial lung biopsies can provide lung tissue, the best way to obtain a good lung sample is through an open lung biopsy (by either classical thoracotomy or thoracoscopy). Nevertheless, in the COPD population, surgery is often precluded due to the poor lung function. In addition to its use as a diagnostic tool, lung resection may be

also therapeutic (Caillot *et al.*, 2001). Lung tissue can also be obtained by percutaneous needle biopsy, with a yield in the range of 50%; thus, negative results do not exclude the diagnosis. Lung biopsy should be performed when there is a doubt in the diagnosis of probable IPA.

Therapy

Currently, voriconazole is the first therapy line. It is also possible to think about combination therapy in severe cases to look for increasing fungicidal activity. However, further clinical studies with combined therapy are needed before the concept can be validated (see treatment section).

Prognosis

Despite treatment, the IPA mortality rate remains high (Lin *et al.*, 2001). In patients with chronic obstructive pulmonary disease, a favourable response (both complete and partial) to treatment has been reported in 56% of patients with pulmonary diseases (Patterson *et al.*, 2000). However, in COPD mortality occurred in 77%, and intensive care unit admission did not seem to modify this poor prognosis. The delay between symptoms and diagnosis may, at least partially, explain this very high mortality (Patterson *et al.*, 2000; Lin *et al.*, 2001).

Although *Aspergillus* colonization is frequent in COPD patients, IPA can occur and prompt diagnosis is important to provide the maximum chance of successful treatment. The first clinical signs are often protracted bronchospam and/or antibiotic-resistant lower respiratory tract infection. In this context, the presence of *Aspergillus* spp. in the sputum must be regarded as the first clue of infection. As the chest radiograph may be normal early on in the course of the disease, a prompt CT scan is required to visualize mild alterations of the lung parenchyma. When possible, bronchoscopy is required to demonstrate mucosal modifications and allow biopsies, as well as BAL. There are insufficient data to support the use of serological tests. If the patient's status is severe, prompt therapy should be implemented to give the patient optimal chance of cure and, thereafter, diagnostic procedures must be conducted to confirm the clinical suspicion of IPA. Although amphotericin B deoxycholate (AmB-d) has been most extensively studied, the results of more recent studies suggest that voriconazole should be the first therapy choice. Patients receiving chronic steroid therapy should be provided with information on how to prevent contact with environmental sources of *Aspergillus* spp. Clearly, more work needs to be carried out in order to better identify *Aspergillus* infection in COPD patients, and to decrease mortality from this devastating disease.

Aspergillus in asthma and cystic fibrosis – Allergic Bronchopulmonary Aspergillosis

ABPA is a disease resulting from a hypersensitivity response to *A. fumigatus*. Although *A. fumigatus* is the primary causative pathogen in ABPA, the disease can be caused by several other members of the *Aspergillus* spp,

including *A. flavus, A. nidulans, A. niger*, as well as other fungi (Nelson *et al.*, 1979; Mastella *et al.*, 2000; Stevens *et al.*, 2003). Patients with asthma and CF are predisposed to developing ABPA, with an estimated prevalence of 1 and 6-8% respectively (Stevens *et al.*, 2003). The pathogenesis of the disease is unknown, although it is postulated that *A. fumigatus* spores are inhaled, trapped in the mucus of the large segmental bronchi, and subsequently germinate to form hyphae, which release antigens to elicit an immune response. The hypersensitivity reaction is characterized by the production of immunoglobulin IgG and IgE antibodies specific for *A. fumigatus*, as well as a rise in total IgE. ABPA is defined through 7 primary diagnostic criteria: episodic bronchial obstruction (asthma), peripheral eosinophilia, immediate scratch test reactivity to *Aspergillus* antigen, precipitating antibodies to *Aspergillus* antigen, elevated serum IgE concentrations, history of pulmonary infiltrates (transient or fixed), and central bronchiectasis. Secondary diagnostic criteria include repeated detection of *Aspergillus* species in sputum samples using stain and/or culture, a history of expectoration of brown plugs or flecks, elevated specific IgE concentration directed against *Aspergillus* antigen, and Arthus reaction (late skin reactivity) to *Aspergillus* antigen. Validated criteria for the diagnosis of ABPA have been established in patients with asthma, but not yet in CF. In CF, diagnosis relies principally on immunological evidence (in particular a positive, immediate skin reactivity to *A. fumigatus* and on the *in vitro* demonstration of specific serum IgE, IgG and precipitating antibodies). The recommended combination of immunological evidence and clinical manifestations is important. Positive immune parameters and elevated total serum IgE levels in a CF patient experiencing increased cough or wheezing, pulmonary infiltrates, or a decrease in pulmonary function that are unresponsive to aggressive therapy (increased antibiotics and bronchodilators) may suggest a diagnosis of ABPA. However, sensitization to *A. fumigatus* is very common in these patients. In fact, in a study of 51 adult CF patients, the immune response to *A. fumigatus* was present in 66% of patients, but only one patient was considered to have ABPA (Geller *et al.*, 1999). Therefore, *A. fumigatus* sensitization, in the absence of clinical features of ABPA, does not suggest an ABPA diagnosis. One of the most relevant problems is the contribution of ABPA to disease progression in CF. This is a poorly documented and little understood issue for which no epidemiological studies have been performed to date. In spite of this, ABPA is considered to be a serious complication in patients with CF. ABPA is associated with higher rates of microbial colonization, pneumothorax, massive haemoptysis, higher IgG serum levels and poorer nutritional status. However, pulmonary lung function decline is not substantially different in ABPA patients compared with non-ABPA patients for any subgroups based on age or disease severity (Mastella *et al.*, 2000).

Treatment

The corticosteroids and antifungal drugs as itraconazole are recommended (see treatment).

In conclusion, ABPA is a pulmonary hypersensitivity disease mediated by an allergic response to *A. fumigatus*. ABPA occurs in up to 8% of CF patients and 1% in asthma. Despite the gold-standard Nelson criteria, diagnosis of ABPA in CF patients remains difficult (Nelson *et al.*, 1979). The wide variation in diagnostic practices between clinics, different estimates of prevalence and a delay in recognition lead to under treatment. The main reason for the difficulties in diagnosis of ABPA and exacerbations in CF patients is the overlap of diagnostic criteria for ABPA with common manifestations of CF. Pulmonary infiltrates, obstructive lung disease and bronchiectasis occur regularly in CF patients, due to the bacterial chronic infection and thus these findings are not specific to ABPA. Furthermore, lung colonization with *A. fumigatus* occurs in 20–25% of CF patients. Therefore, as stated in the most recent consensus document on diagnosis and therapy of ABPA in CF patients, serological findings should contribute strongly to the confirmation or exclusion of clinically suspected ABPA. Treatment of APBA should consist of a combination of corticosteroids and itraconazole (A-I) (Walsh *et al.*, 2008).

Prophylaxis aspergillosis (in LT patients)

Nowadays, there is not a universal approach about antifungal prophylaxis in patients with respiratory diseases at high risk of aspergillosis, only in LT recipients. Several prophylactic strategies with antifungal drugs have been reported to result in a decreased incidence and mortality of fungal disease in LT recipients (Covarrubias and Milstone, 2005; Husain *et al.*, 2006b; Magill and Dropulic, 2006). However, data are limited, and there is a considerable variation in antifungal prophylaxis practices among LT centres throughout the world. The majority of LT programmes are using universal antifungal prophylaxis in the postoperative period; about 30% use a pre-emptive approach for patients with pre- and/or post-transplant fungal airway colonization. It is clear that there is a considerable uncertainty to which approach (prophylaxis or pre-emptive therapy) is most appropriate, which agent is the best, and what duration of prophylaxis or pre-emptive therapy is needed. Antifungal prophylaxis in LT recipients should be taken into account, as well as the incidence of colonization, anastomoses healing, chronic rejection, and the time of LT, thus providing a rationale for the duration of therapy.

To prevent IPA, multiple strategies and antifungal drugs have been utilized, such as oral itraconazole, voriconazole or aerosolized AmB used alone or in combination. Aerosolized medication regimens are an attractive option, as drug interactions and systemic toxicities are likely to be limited (Drew, 2006). Several centers have reported on the safety of aerosolized AmBd with a variety of dosing regimens (Reichenspurner *et al.*, 1997; Calvo *et al.*, 1999; Monforte *et al.*, 2003), and others with aerosolized amphotericin B lipid formulations (Palmer *et al.*, 2001; Drew *et al.*, 2004; Monforte *et al.*, 2005; Lowry *et al.*, 2007). Our institution has used aerosolized AmBd as part of the post-LT protocol since 1994 (Calvo *et al.*, 1999). For the past three years we have also been using

amphotericin B lipid complex (ABLC), with the same respiratory tolerability and safety that aerosolized AmBd, but ABLC use is more comfortable for long periods of time (50 mg inhaled/weekly), and patients have better adherence to treatment. Regarding oral prophylaxis, a recently study (Husain *et al.*, 2006a) has been published. This study examined the efficacy and toxicity of a strategy of universal *de novo* antifungal prophylaxis with voriconazole compared to targeted antifungal prophylaxis. The main finding of this study was that the overall rate of aspergillosis at 1 year decreased to 1.5% with universal voriconazole prophylaxis as compared to 23.5% with a targeted prophylaxis strategy. Interestingly, the rate of *Candida* colonization, particularly non-*albicans* species, in the voriconazole group was significantly higher (Husain *et al.*, 2006a). In the voriconazole prophylaxis cohort, 27% of the LT recipients had normal liver enzymes throughout the course of the study. The main handicap of this azole therapy is the strong interaction with immunosuppressors that forces to monitoring calcineurin inhibitors in order to avoid toxicity or rejection. Other interesting finding was that universal voriconazole prophylaxis did not increase the rate of non-*Aspergillus* fungal infections (specially, zygomycosis).

Newer azoles (voriconazole, posaconazole) with predictable bioavailability should be preferred over the azole (itraconazole) with erratic bioavailability. Available echinocandins (caspofungin, micafungin, anidulafungin) may have an important role in antifungal prophylaxis because of their antifungal profile, pharmacokinetics and security; however, they are expensive and need intravenous administration. Lipid preparations of AmB appear to be ideal for inhalational administration; however, there are not rigorous pharmacokinetic studies in LT recipients to determine the appropriate dose and schedule of their administration. Monforte and colleagues have demonstrated that aerosolized AmBd and lipid preparations of AmB are safe and achieve high concentrations in BAL fluid for the first 24 hours and 14 days, respectively, following nebulization (Monforte *et al.*, 2003, 2005). These lipid formulations let a delayed administration (every 7-14 days), which is rebounded in a better accomplish by patient. Although the incidence of IFI seems to be reduced with aerosolized AmB prophylaxis, the efficacy of this approach has not been determined in a large prospective clinical trial. Furthermore, without detectable levels of AmB in the circulation, extrapulmonary fungal infections may not be prevented by this strategy. Besides, it is important to take into consideration the type of delivery systems used for inhaled drugs (Corcoran *et al.*, 2006; Hagerman *et al.*, 2006). In addition, contamination of the nebulization systems used in the prophylaxis with AmB nebulized in LT has been described (Monforte *et al.*, 2005).

Another question is how long should be prophylaxis maintained? The majority of centers agree to apply universal prophylaxis during first period post transplant (3 months) after this time, each center use a tailored prophylaxis. Besides, it is recommended to use nebulized antifungal prophylaxis and/or preemptive therapy with antifungal agents (voriconazole) in patients with chronic rejection and respiratory samples positive for *Aspergillus*, even without clinical

or radiological signs, mainly in single LT patients due to the high risk of IA. This preemptive treatment should last for at least 6 months, the time period over which colonization has been shown to precede disseminated infection, and in some cases for life.

Treatment

It is recommended to start antifungal therapy promptly in a patient who shows clinical signs and symptoms compatible with IA. Considering the association between early onset of systemic antifungal treatment and improved clinical outcome in many patients, the diagnosis of IA will never be proven because treatment has led to complete or partial regression of these findings, or because the patient dies and autopsy is refused. Although prospective studies have never been conducted on the optimal time to start systemic antifungal therapy after the appearance of the first clinical signs and symptoms, it is generally accepted that early onset of treatment with systemic antifungal agents results in significantly higher survival rates (von Eiff *et al.*, 1995; Stevens *et al.*, 2000a; Bohme *et al.*, 2003). The mainstay of treatment is drug therapy supplemented, when indicated or feasible, by reversal of underlying immunosuppression, surgery and, rarely, immune modulation. The drugs currently available that are effective against *Aspergillus* species are amphotericin B (including liposomal forms), itraconazole, voriconazole, posaconazole, caspofungin, anidulafungin and micafungin. Pharmacological considerations may be important for the clinical choice of an antifungal agent for treatment of IA. *In vitro* susceptibility testing may not be reliable to predict clinical response in patients with IA, because for many antifungal drugs, a standardised susceptibility testing system for *Aspergillus* spp. has not yet been established. However, *Aspergillus* may occasionally be resistant to some of these drugs; for example, *A. terreus* has a high incidence of resistance to AmB, and *A. lentulus* has a wide and extensive panresistance to AmB, voriconazole and caspofungin. More recently multiple triazole resistant *A. fumigatus* isolates have been described (Mellado *et al.*, 2007; Verweij *et al.*, 2007). In most cases, however, failure of these drugs is caused not by drug resistance but by the immune status of the patient. Length of therapy has not been established and it is not well standardized, but many courses continue for 10 to 12 weeks or several weeks after clinical and radiographic resolution.

This expanded antifungal armamentarium offers the clinician a number of new therapeutic choices but also raises questions about where each new agent fits clinically. Clinicians must understand the advantages and limitations of each drug and drug class in order to optimally utilize these agents to manage patients' invasive mycoses. Excellent, comprehensive reviews of the pharmacology, microbiology, and mechanisms of action of these agents have been published elsewhere (Ashley *et al.*, 2006; Catalan and Montejo, 2006; Pachon *et al.*, 2006; Petrikkos and Skiada, 2007). Furthermore, recently the new guidelines of the Infectious Diseases Society of America (IDSA) for treatment of aspergillosis have been published (Walsh *et al.*, 2008) and replace the practice guidelines for

Aspergillus published in 2000 (Stevens *et al.*, 2000a). This new document summarizes the current evidence for treatment of different forms of aspergillosis. We will refer to the latter in the following paragraphs. Finally, outcome of the IFI is associated with immune reconstitution and neutrophil recovery in case of neutropenia associated to transplantation. This section presents (Table 4.1) recent clinical evidence on the efficacy and limitations of these new agents, and includes our personal opinions.

Table 4.1. Antifungal agents against *Aspergillus* infections and other opportunistic and systemic mycoses

Agent	Class	Spectrum and Clinical Indications	Mechanism of action	Advantages	Limitations	Preferential toxicity and adverse reactions	Cost
Amphoter-icin B deoxycholate (AmBd)	Polyene	*Candida*, *Aspergillus*, Zygomycetes, and other opportunistic mycoses.	Destabilizes the fungal cell membrane. Binds to the sterol ergosterol incorporated in the fungal cell membrane	Broad spectrum of activity, few resistant fungi.	Dose-limiting renal and infusion- related toxicity; poor efficacy in immuno-suppressed hosts.	Infusion-related and/ or Nephrotic: +++	$
Lipid formulations of amphotericin B (L-AmB, ABLC, AmB-CD)		Broad spectrum of activity, similar to parent compound.	which creates pores in the membrane and leads to depolarization of the membrane with subsequent cell leakage. In mammalian cells, polyenes bind cholesterol	Less toxicity than AmB. Possibility of combination with other antifungal drugs and Efungumab (Mycograb®)	Renal, infusion-related, and other acute toxicities (with ABCD having the greatest toxicity, and L-AmB having the least); few primary treatment studies; expensive.	+ (L-AmB) ++ (ABLC, AmB-CD)	$$$$ (L-AmB) $$$ (ABLC, AmB-CD)
Voriconazole	Exten ded spectrum Azole (second generation triazole)	*Aspergillus* and other Moulds: Serious fungal infections caused by *Scedosporium apiospe rmum* and *Fusarium*	Interfere with sterol synthesis via inhibition of CYP-dependent C-14-α demethylase, a fungal CYP	Survival advantage vs AmB for invasive aspergillosis (recommended primary therapy for most patients);	Extensive drug interactions; visual, liver, skin toxicities; azole cross-resistance in yeasts. Intravenous formulation should be	Hepatic: ++ ++	$$ / $$$ (or / iv) $$$

	spp. in patients intolerant of or refractory to other therapy;	enzyme important in converting lanosterol to ergosterol	intravenous and oral delivery.	restricted in severe renal failure.		
	Candida, especially non-*albicans* and other yeasts.		Possible benefit (both) in combination therapy for aspergillosis and other moulds infections.	Possible need to monitor plasmatic levels for efficacy.		
Posaconazole	Zygomycetes and other emerging fungal infections; salvage therapy and prophylaxis of invasive *Aspergillus* and *Candida* infections in high-risk patients with leukaemia or HSCT, not in SOT patients.		Activity against Zygomycetes; well tolerated in trials.	Oral suspension only; azole cross-resistance.		
Caspofungin, Echinmicafungin, ocandin anidulafungin	*Candida* spp. (candidemia, invasive candidiasis), salvage therapy for *Aspergillus*. in patients who are refractory to or in tolerant of other therapies (*Aspergillus* regulatory approval only for caspofungin) Empirical therapy for presumed fungal infections in febrile neutropenia	Inhibition of β-(1,3) glucan synthesis via inhibition of β-(1,3) glucan synthase. Fungal cell wall is mostly polysaccharides, and glucans are the most abundant polymers in fungal cell walls. Glucan synthase catalyzes polymerization of	Extremely well tolerated; anidulafungin more effective vs fluconazole in one study of candidemia; micafungin equal effective vs amphotericin B in one study of candidemia but less toxicity; micafungin non inferior to a standard dosage of caspofungina for treatment of candidemia and other forms of invasive	Mould activity targeted to *Aspergillus*; potential cyclosporine interaction for caspofungin; expensive. Non activity on the genera *Cryptococcus*. *C parapsilosis* breakthrough candidemia. Pending *in vitro* susceptibility study methodology.	Infusion-related and /or Hepatic: + + +	$$$$ (caspofungina) $$$ (micafungin, anidulafungin)

		(only caspofungin) Prophylaxis of *Candida* infections in patients undergoing HSCT (micafungin)	these polysaccharides. Inhibition of this unique enzyme ultimately leads to increased cell wall permeability & lysis of the cell.	candidiasis; anecdotal benefit in combination therapy for aspergillosis. Possibility of combination with other antifungal drugs (triazole).			
Others: Flucytosine	Fluorinated analogue of cytosine	Treatment of serious infections caused by susceptible strains of *Candida* and/or *Cryptococcus* spp., principally in combined antifungal treatment with amphotericin B or azoles	Transported intracellularly by cytosine permease. Converted to fluorouracil via cytosine deaminase, and subsequently to 5-fluorouridine triphosphate, which is incorporated into fungal RNA and interferes with protein synthesis. The flucytosine intermediate also inhibits thymidylate synthase, and interferes with DNA synthesis	In combination, incremented clearance of *Cryptococcus* in CSF; historical and recent studies are favourable for combination antifungal therapy in cryptococcal meningitis; disposable in oral and intravenous formulations	No commercialized in all the countries; gastrointestinal intolerance very frequently; iv solution if oral route contraindicated; monitor serum creatinine and blood counts at regular intervals; precaution in patients with moderate/severe renal or hepatic dysfunction	Hematologic: +++ Hepatic: ++	$ $
Terbinafine	Allyl amine	Any species of *Candida*, *Cryptococcus* and mycelial fungi (specially *Aspergillus*, *Scedosporium* and dematiaceous moulds);	Blockade of ergosterol syntesis by escualenoepoxidase inhibition, a fungal enzyme	Good absorption and adequate corporal distribution with accumulation in fat tissues; *in vitro* studies are favourable for	Gastroin-testinal intolerance is common; important interactions with cyclosporine and rifampicine; precaution in patients with		

use in combination with azoles in cases of multiresistant fungal pathogen in immunosuppressed hosts; topical or oral therapy of dermatomycoses	use in combination strategy versus panresistant fungi	moderate/severe renal or hepatic dysfunction

Plus signs indicate degree of toxicity: +, mild; ++, moderate; +++, severe; AmB-CD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; AmBd, deoxycholate amphotericin B; AmB-L, liposomal amphotericin B. $: low cost; $$: intermediate cost; $$$: high cost; $$$$: very expensive. CSF: cerebrospinal fluid; or: oral route; iv: intravenous route; SOT: solid organ transplantation; HSCT: hematopoietic stem-cells transplantation

AmBd has been the gold standard antifungal therapy for opportunistic mycoses for more than four decades. However, it is associated with unacceptable toxicities and is frequently in effective, particularly in those patients with advanced immunosuppression. In addition, outcomes of salvage therapy following progression of infection or toxicity after initiation of AmB are extremely poor. For these reasons, antifungal agents with better tolerability and efficacy have been urgently needed. In the last decade, several new agents have been introduced, including the lipid formulations of AmB, which significantly reduce the toxicity of AmB-d and enhance its therapeutic index; extended-spectrum azoles, with improved activity against moulds; and echinocandins, a new class of antifungal with a novel mechanism of action against the cell wall. Despite the expansion of antifungal armamentarium over the past decade, the mortality rate for IFI remains high in severely immunocompromised patients. Three classes of antifungals (polyenes, extended-spectrum azoles, and echinocandins) are now available for treating systemic fungal infections. Guidance for the appropriate use of this expanded variety of antifungals may come from recent clinical trials. Extended-spectrum azoles have excellent *in vitro* activity against *Aspergillus* and have been shown to improve clinical outcomes. These new agents offer less toxicity and potentially improved efficacy in these difficult infections.

There are few randomized trials on the treatment of IA. AmB has been the main antifungal drug in use for *Aspergillus* infection, but voriconazole is now considered a first-line therapy and is being used increasingly. In a largest randomized, controlled, prospective trial comparing voriconazole with conventional AmB in 277 immunocompromised patients (including 79 HSCT and 14 solid organ transplant recipients) with confirmed or probable invasive aspergillosis, the use of voriconazole was associated with a greater likelihood of complete or partial response at 12 weeks, lower mortality, and less likelihood of needing other drugs (Herbrecht *et al.*, 2002). Voriconazole showed, in patients

with IA, superiority in clinical response, survival, and toxicity. These results have placed voriconazole as the preferred drug for primary therapy of IA. Voriconazole is recommended for the primary treatment of IA in most patients (scored as category/grade A-I for the quality of evidence) (Walsh *et al.*, 2008). Although IPA accounts for the preponderance of cases treated with voriconazole, this drug has been used in enough cases of extrapulmonary and disseminated infection to allow us to infer that voriconazole is effective in these cases (Schwartz *et al.*, 2005).

A randomized trial comparing two doses of AmB-L showed similar efficacy in both arms, suggesting that liposomal therapy could be considered as alternative primary therapy in some patients (Cornely *et al.*, 2007) (A-I). For salvage therapy, agents include lipid formulations of AmB (A-II), posaconazole (B-II), itraconazole (B-II), caspofungin (B-II), or micafungin (B-II). Salvage therapy for IA poses important challenges with significant gaps in knowledge. In patients whose aspergillosis is refractory to voriconazole, a paucity of data exist to guide management. Therapeutic options include a change of class using an AmB formulation or an echinocandin, such as caspofungin (B-II); further use of azoles should take into account host factors and pharmacokinetic considerations. Refractory infection may respond to a change to another drug class (B-II) or to a combination of agents (B-II).

Data on the efficacy of posaconazole for clinical treatment of patients with IA are sparse. A study that analyzed the efficacy of posaconazole in patients with refractory or intolerant IA was recently published. Complete response was noted in 7% versus 9%, partial response in 36% versus 16%, stable disease in 9% versus 8%, and non-response in 36% versus 60% of patients compared with controls (Walsh *et al.*, 2007). Limited data are available on the use of therapeutic drug monitoring, but in the latter study, improved efficacy occurred with higher posaconazole drug levels. It is an inhibitor of CYP3A4, which is responsible for a considerable spectrum of drug-drug interactions (Wexler *et al.*, 2004); however, these are less pronounced than those observed for voriconazole.

Micafungin and anidulafungin have activity against *Aspergillus* species but are not approved for that indication, and optimal doses for aspergillosis have not been established. As with caspofungin, no results are available from prospective, randomised studies on the use of micafungin for the treatment of IA. There is a multinational nonrandomised trial on 331 patients with IA treated with micafungin as primary treatment or as salvage therapy. More than 30% of patients showed partial or complete response. Combination with other antifungals had no obvious benefit, and higher daily doses were not reported to be associated with more favourable response rates. The profile of safety and security was similar to caspofungin (Denning *et al.*, 2006). Anidulafungin is the third licensed echinocandin antifungal. It is not yet approved for treatment of IA in any country. Safety analyses have been performed in neutropenic children at risk for IFI (Benjamin, Jr. *et al.*, 2006) and in adults with IA treated with anidulafungin

in combination with AmB-L (Herbrecht *et al.*, 2004), but data on therapeutic efficacy in patients with documented aspergillosis have not been published to date.

Tracheobronchitis

There are no good studies reporting results of strategies for treating *Aspergillus* tracheobronchitis. A common practice at some centres is to use aerosolized AmB combined with some systemic antifungal therapy, typically intravenous AmB or an oral azole (Kotloff *et al.*, 2004; Singh and Paterson, 2005). Anecdotal reports of success with single drug or various combination drug regimens have been described (Kramer *et al.*, 1991; Monforte *et al.*, 2003; Solé *et al.*, 2005). Stents may need to be placed for postinfectious bronchial stenosis.

Voriconazole and itraconazole have been used successfully in the treatment of this form of PA (Denning *et al.*, 2002). Direct instillation of AMB has been also administered in association with systemic therapy (Boettcher *et al.*, 2000; Hadjiliadis *et al.*, 2000).

In the recent guidelines of IDSA (Walsh *et al.*, 2008), voriconazole is recommended as initial therapy in the treatment of tracheobronchial aspergillosis (B-II). Little experience is available with caspofungin or other echinocandins in treating this infection. Because the use of AmBd may result in increased nephrotoxicity in association with calcineurin inhibitors, a lipid formulation of AmB is recommended if a polyene is considered in the lung transplant recipient (B-III). Bronchoscopic evaluation is the most important aspect of initial diagnosis; CT will assess the lack of progression to the remainder of the pulmonary tree. Reduction of immunosuppression, where possible, is an important element in improving therapeutic outcome. Aerosolized AmB-d or lipid formulations of AmB may have some benefit for delivering high concentrations of polyene therapy to the infected (often anastomotic) site; however, this approach has not been standardized and remains investigational (C-III).

***Aspergillus* infection in patients with COPD**

Although published data specific for COPD patients are lacking, it seems reasonable to recommend a thoracic CT scan as soon as pulmonary involvement is suspected. In case of compatible lesion(s), treatment must be initiated promptly. However, it is reasonable to apply antifungal therapy as in other lung diseases. Currently, voriconazole is the first line therapy, and it is also possible to think about combination therapy in severe cases when looking for increasing fungicidal activity. However, further clinical studies with combined therapy are needed before the concept can be validated.

Although antifungal drugs represent the first choice in treatment of IA, eradication is rare as residual lesions are often present, and therefore potential recurrence is possible in the case of a new increase of immunodepression. To

obviate IPA relapse, some authors have suggested the resection of residual lesion(s) in addition to medical therapy (Walsh *et al.*, 2008). In COPD patients, surgical resection of IPA is generally excluded due to their poor pulmonary function. Surgery could theoretically be considered in selected cases and minimal resection due to the functional defect (Habicht *et al.*, 2000; Matt *et al.*, 2004). However, no recent study has been published to support this strategy.

Allergic Bronchopulmonary Aspergillosis

The corticosteroids, taken initially in high doses, and then over a long period of time in lower doses, may prevent progressive lung damage. Although corticosteroid therapy is the mainstay of therapy for ABPA, there are only few studies of corticosteroid therapy for ABPA, which, in addition, have involved small numbers of patients and were neither double-blind nor controlled. Nevertheless, the current findings support the usefulness of corticosteroids in the management of acute ABPA, with improved pulmonary function and fewer episodes of recurrent consolidation. However, chronic administration of corticosteroids causes severe immune impairment and multiple metabolic abnormalities.

The antifungal drugs as itraconazole or newer azoles usually are used in addition to corticosteroids to help eliminate the fungus from the lung. To avoid side effects of chronic administration of corticosteroids, alternative approaches to management of ABPA have been developed. An example of such an approach is to eradicate *Aspergillus* species from the airways by using itraconazole as a corticosteroid sparing agent. The mechanism of this effect is to diminish the antigenic stimulus for bronchial inflammation.. Two double blind, randomized, placebo-controlled trials for ABPA demonstrated that itraconazole (200 mg twice daily orally for 16 weeks) resulted in significant differences in ability to ameliorate disease, as assessed by the reduction in corticosteroid dose, increased interval between corti-costeroid courses, eosinophilic inflammatory parameters, and IgE concentration, as well as improvement in exercise tolerance and pulmonary function (Stevens *et al.*, 2000b; Wark *et al.*, 2003). Similar benefits of itraconazole have been observed in patients with cystic fibrosis and ABPA (Skov *et al.*, 2002). Other azoles (voriconazole and posaconazole) have not been studied in this context. The benefits of short-term corticosteroid treatment of ABPA include reduced frequency of acute exacerbations, preservation of pulmonary function, and improved quality of life. However, the long-term adverse effects of corti-costeroid therapy may result in profound immunosuppression and debilitating metabolic abnormalities, including diabetes mellitus, hyperlipidemia, and osteoporosis. Corticosteroid-induced immuno-suppression may very rarely result in progression of ABPA to invasive pulmonary aspergillosis. The benefits of the addition of itraconazole outweigh the risks of long-term administration of high-dose prednisone.

Owing to the fact that the lung damage may worsen gradually without causing any noticeable changes in symptoms, chest X-rays, pulmonary function tests, levels of eosinophils in the blood, and amounts of immunoglobulin E

antibody are regularly monitored during treatment. As the disease is controlled, the eosinophil and antibody levels usually fall, but they may rise again as an early sign of flare-ups.

Combined antifungal treatment

Because of the increasing incidence of invasive *Aspergillus* infections and their associated morbidity and mortality, better therapeutic approaches are needed to treat IA. The availability of new antifungal agents with unique mechanisms of action and improved tolerability has widened the possibilities for the use of combination antifungal therapy for difficult-to-treat opportunistic mycoses (Chamilos and Kontoyiannis, 2006). An important advance in the antifungal armamentarium is the availability of the newer broad spectrum azoles voriconazole and posaconazole, which have been studied for IA (Raad *et al.*, 2007; Walsh *et al.*, 2007). Researchers have studied the echinocandin class as a salvage therapy in IA, and results have been encouraging, making potential combination therapy with these drugs attractive (Maertens *et al.*, 2004; Denning *et al.*, 2006; Maertens *et al.*, 2006).

Generally, for *in vitro* studies, combinations of AmB and azoles against *Aspergillus* spp show indifference or antagonism (Denning *et al.*, 1992; Meletiadis *et al.*, 2006). Studies of AmB in combination with echinocandins have ranged from indifference to synergy (Arikan *et al.*, 2002). Combinations of the echinocandins with triazoles range from synergistic activity to indifference, but importantly, no antagonism has been reported (Perea *et al.*, 2002). Animal models of IA generally confirm results predicted by *in vitro* combination tests. Antagonism is often seen in the combination of AmB plus azoles (Polak *et al.*, 1982; Clemons *et al.*, 2005). Recently, several important *in vivo* studies evaluating newer azoles and echinocandin combinations have suggested that combination therapy is superior to single therapy by clinical parameters such as survival, GM antigenemia, and reduced colony counts in tissues (Kirkpatrick *et al.*, 2002; Petraitis *et al.*, 2003). Importantly, no antagonism was demonstrated in these studies. Other studies have evaluated AmB and echinocandin combinations with favourable results (Clemons *et al.*, 2005), showing that combination therapy resulted in reduced kidney burden of organisms, increased survival, and improved histopathologic findings with combination therapy. However, a study by Petraitis *et al.* (1999) found neither synergy nor antagonism with the combination of micafungin and AmB. These *in vivo* studies demonstrated that combinations of expanded-spectrum triazoles plus an echinocandin or AmB plus an echinocandin have clinical potential and warrant further clinical investigation.

Antifungal combinations are increasingly used in clinical practice to improve outcomes for refractory mycoses because of the suboptimal efficacy of current antifungal agents. However, the use of this therapy is largely governed by empiricism, especially in patients with invasive mould infections, given that there is a tremendous need to improve outcomes (Kontoyiannis and Lewis, 2004). The

benefits of combination antifungal therapy have been difficult to prove for IFI other than cryptococcal meningitis. The recent introduction of several new antifungal agents has renewed interest in studying those combinations for difficult-to-treat aspergillosis. The majority of the studies evaluating antifungal combinations are still performed in the laboratory or using animal models of infection. The methods used to assess combined antifungal effects *in vitro* and in animals are poorly standardized, and there is little evidence that data generated from these studies can be translated in treating human mycotic infections, specially in patients with SOT and HSCT (Leather and Wingard, 2006; Munoz *et al.*, 2006). Despite the empiricism of combination antifungal therapy, certain principles help in guiding the use and study of these regimens (Johnson and Perfect, 2007). In view of the evolving epidemiology of IFI, combination antifungal therapy could be most valuable in preemptive management of carefully selected high-risk patients; however, this should be studied in appropriate trials.

Most information on combination therapy for the treatment of aspergillosis is derived from retrospective case series and reviews, especially in haematological patients (Aliff *et al.*, 2003; Kontoyiannis *et al.*, 2003) or HSCT recipients, which do not allow for appropriate critical analyses. There is only a small number of clinical studies which report on the use of combination therapy of expanded-spectrum triazoles plus echinocandins or AmB preparations plus echinocandins for IA (Marr *et al.*, 2004). Most studies are limited by retrospective evaluation, use of historical controls, and use of combination therapy as salvage treatment. Nevertheless, these studies, reviewed below, bring attention to toxicity concerns and important design issues that are necessary for future successful combination therapy clinical trials.

No single randomized study on antifungal combination therapy in SOT patients has been performed (Baddley and Pappas, 2005). Existing information does not support the use of combination therapy in invasive candidiasis in SOT patients. Indeed, initial combination therapy with AmB and flucytosine is recommended for SOT patients with central nervous system cryptococcosis, mainly with increased white blood cell counts in the cerebrospinal fluid or with altered mental status. No impact over the outcome was observed with combination therapy in *Scedosporium* infections in SOT patients. The combination of voriconazole and terbinafine may be an attractive option for *S. prolificans* infections. A prospective study of voriconazole plus caspofungin as initial therapy for IA in SOT patients found that combination therapy was independently associated with reduced mortality in patients with renal failure and in those with *A. fumigatus* infection, even when adjusted for other predictive factors of mortality in the study population (Singh *et al.*, 2006a). This study was important because it included many solid organ transplant recipients, but it was limited by the use of a historical control group.

Due to the previously mentioned limitations, responses with combination therapy must be interpreted with caution. Importantly, these studies suggest that

combination therapies are relatively safe and lack antagonism, but superiority of combination therapy has not yet been determined. Moreover, drug interactions and health care costs associated with combination antifungal therapy have not been fully elucidated. Considering the *in vitro*, *in vivo*, and available clinical data for AmB plus echinocandins and triazoles plus echinocandins, these important clinical research questions require a randomized clinical trial. In summary, combination therapy should be considered for severe forms of invasive fungal infections and IA in SOT patients; however, multicenter studies of such patients are urgently needed. A large clinical trial studying combination therapy for IA is feasible but would require a collaborative international effort and substantial support from both the pharmaceutical industry and clinicians.

Surgery

Surgical debridement, excision of localized infections (lung, sinuses, eye, brain, soft tissue, and bone), and removal of infected intravenous catheters is required in patients with localized infections to delay or stop dissemination. Surgical resection of pulmonary lesions due to *Aspergillus* species can provide a definitive diagnosis and can potentially completely eradicate a localized infection. Surgery is also used for the control of massive haemoptysis if the cause is focal disease. It has also been suggested that, for patients who have one or two focal lesions, surgery with resection added to primary medical therapy may improve outcomes (Habicht *et al.*, 1999). The theory is that radical surgical removal of necrotic and poorly perfused lung tissue will clear IPA more quickly. This approach is used more commonly in infections caused by *Mucor* than in those caused by *Aspergillus*. Multiple studies have shown that resection can be performed with acceptable mortality, but the exact setting in which surgery should be performed is unclear. Data from controlled clinical trials is not available, and the effect on patient survival must be critically reconsidered in the light of new diagnostic tools for early detection of IA and the broad spectrum of effective new antifungal agents available today.

Decisions concerning surgical therapy should be individualized to account for a number of variables, including the degree of resection (e.g., wedge resection vs. pneumonectomy), potential impact of delays in transplantation, comorbidities, performance status of the lung diseases, the goal of immunosuppressive therapy, and unilateral versus bilateral lesions.

Immunotherapy and adjunctive drug treatment

Decreasing the level of immunosuppression is an important factor for a successful treatment of IPA. Acute or chronic rejection of the transplanted organ and persistent neutropenia are two of the most important variables for poor outcome in IA. Failure to recover from neutropenia is often associated with a fatal outcome of IPA, and withdrawal of corticosteroids or reduction of dosage is often critical for successful outcome in IA. The failure to reduce an

immunosuppressive dosage of systemic corticosteroids usually results in relentless IFI. However, because a control of underlying diseases such as organ rejection- may only be achieved by intense immunosuppression, corticosteroid-sparing immunosuppressive strategies are being used increasingly.

Cytokines, such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and IFN-γ, also augment functional properties of phagocytic cells through upregulation of chemotaxis, phagocytosis, oxidative metabolism, and/or degranulation of neutrophils. Granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and IFN-γ upregulate phagocytosis and the respiratory burst of monocytes and macrophages (Stevens, 2006). The clinical data suggest a potential role of IFN-γ in selected hosts for prevention or treatment of invasive aspergillosis (Ezekowitz, 2000). Although clinical data supporting its use specifically for aspergillosis are sparse, IFN-γ is widely used for prevention of bacterial and fungal infections in patients with chronic granulomatous disease (CGD) (The International Chronic Granulomatous Disease Cooperative Study Group, 1991). Individual case reports suggest a role for IFN-γ as adjunctive antifungal therapy for IA in immunocompromised nonneutropenic patients, particularly those with CGD.

Fungi have developed complex and coordinated mechanisms to survive in the environment and in the mammalian host. Fungi must adapt to "stressors" in the host (including scarcity of nutrients, pH, and reactive oxygen and nitrogen intermediates) in addition to evading host immunity. Awareness of the immunopathogenesis of fungal infections has paved the way to promising strategies for immunotherapy. These include strategies that increase phagocyte number, activate innate host defense pathways in phagocytes and dendritic cells, and stimulate antigen-specific immunity (e.g., vaccines). Immunotherapy must be tailored to specific immunocompromised states. Challenges exist in bringing promising immunotherapies from the laboratory to clinical trials.

Conclusion

Aspergillosis is a large spectrum of diseases caused by members of the genus *Aspergillus*. The principal entity is invasive aspergillosis (IA). However, *Aspergillus* species can also produce a wide range of chronic, saprophytic, and allergic conditions. This review highlights the spectrum of *Aspergillus* infections in several respiratory diseases, mainly invasive aspergillosis in lung transplantation recipients and COPD, and also ABPA in asthma and cystic fibrosis (CF) patients. One issue of IFI is the difficulty in making a definitive diagnosis; the treatment is sometimes delayed or is not prescribed (post-mortem diagnosis). Serological and molecular detection of *Aspergillus* antigens or fungal DNA, in blood and/or BAL samples, may improve the diagnosis of pulmonary invasive aspergillosis, but the sensitivity is variable and more studies are needed. ABPA poses a high diagnostic difficulty in CF patients, because there is an overlap between the diagnostic criteria of ABPA and common manifestations of CF. In

fact in CF, diagnosis relies principally on immunological evidence; however, in asthma, bronchiectasis, and CF, a combination of immunological evidence and compatible clinical findings for diagnosis of ABPA is recommended. Another pendent issue is antifungal prophylaxis for respiratory diseases at risk of invasive aspergillosis; it is unknown which is the best agent or the time duration.

The last decade has seen the development of newer agents to treat IFI, which have revolutionised the care of patients with invasive mycoses. Treatment combining AmB preparations, newer antifungal drugs, early surgical resection of infected tissue, and discontinuation or modulation of immunosuppressive treatment may be necessary in selected patients and in certain occasions, and all of them may improve prognosis of IFI. However, there are two main handicaps in the management of IFI in respiratory diseases: firstly, to establish an early diagnosis; secondly, delays in applying early treatment with antifungal drugs. The important work for the next years will be the development of new and more precise early diagnostic tools, and a better design of multicentre evaluations of diagnostic methods and therapeutic regimens that are currently available.

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CHAPTER - 5

FUNGAL INFECTIONS OF THE CENTRAL NERVOUS SYSTEM

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Introduction

There are four main causes of infections of the central nervous system (CNS). These include bacterial, viral, fungal, and protozoal agents. Bacterial infections can be caused by pyogenic organisms, or may be due to mycobacteria or spirochetes. Bacterial infections, particularly those due to pyogenic organisms, may lead to meningitis, brain abscess, epidural or subdural abscesses. Viral infections may also lead to meningitis, or can cause encephalitis, or myelitis. Protozoal infections that may affect the brain include toxoplasmosis, malaria, and amoebiasis.

While the majority of infections in the CNS are caused by bacteria and viruses, fungi are increasingly being recognized as important pathogens. Two main factors contribute to this steady increase of fungal infections, namely the widespread use of antibacterial agents and a rapid increase in the numbers of the immuno compromised population. Fungal infections often follow the use of antibiotics, which kill nonpathogenic as well as pathogenic bacteria, thereby providing a free field in the body for fungal invasion. Modern therapeutic modalities such as cancer chemotherapy and organ transplantation have greatly increased the immunocompromised population who are also at a risk.

With improved therapies, patients with cancer survive longer. Both the acute complications of intensive therapies and the risks of chronic immunosuppression have led to an increased incidence of CNS infections. The presentation and

course of common infections may be different from those in patients without cancer, and new syndromes related both to the underlying diseases and to their treatment have complicated the differential diagnosis (Pruitt, 2003, 2004).

New fungal pathogens are being discovered and new syndromes are being described for both known and emerging fungal pathogens. Opportunistic fungal infection occurs when a fungus that is normally non-pathogenic in normal hosts, enters a compromised host, as in patients with acquired immunodeficiency disease (AIDS), resulting in a disease process (Cunha, 2001).

Fungal infections of the CNS are life threatening, and almost always a clinical surprise. Their presentation is subtle, often without any diagnostic characteristics, and they are frequently mistaken for tuberculous meningitis, pyogenic abscess, or brain tumor. Granulocytopenia, cellular and humoral mediated immune dysfunctions are predisposing factors to the development of CNS infections in immunosuppressed patients.

Environmental factors in tropical countries such as India play a significant role in the pathogenesis of CNS fungal infection. There are several published large series and reviews from India about various fungal organisms involving the CNS, including aspergillosis, zygomycosis, and phaeohyphomycosis (Murthy *et al.,* 2001; Nithyanandam *et al.,* 2003; Sundaram *et al.,* 2006; Shankar *et al.,* 2007)

In general, fungal invasion of the CNS may produce one or more of the following clinical syndromes:

- Sub-acute or chronic meningitis.
- Encephalitis.
- Parenchymal brain abscesses or granulomas.
- Vasculitis.
- Vascular thrombosis leading to infarction or stroke, or myelopathy.

The most common pattern of the disease is basal meningitis or intraparenchymal abscesses due to fungal pathogens.

Fungal diseases in the brain are usually secondary to infections elsewhere in the body, particularly the lungs, less often in other extracranial sites. In the vast majority of the cases, spread of infection is through the bloodstream. Intracranial seeding occurs during dissemination of the organism or only occasionally by direct extension from an area anatomically adjacent to the brain.

There are two main groups of fungal organisms that can affect the CNS:

I. Pathogenic fungi: Capable of infecting healthy hosts

1. *Cryptococcus neoformans*
2. *Histoplasma capsulatum*
3. *Coccidioides immitis*

4. *Paracoccidioides brasiliensis*
5. *Sporothrix schenckii*
6. *Blastomyces dermatides*

II. Opportunistic fungi: Usually cause infections in immune compromised host.

1. Aspergillosis
2. Zygomycosis (Mucormycosis)
3. Cerebral Phaeohyphomycoses
4. Candidiasis (*C. albicans, C. tropicalis, C.lusitaniae, C. viswathii*)
5. Penicillioses

All the major fungal pathogens can produce meningitis. On a fungus-specific basis, meningitis ranges from the relatively common cryptococcal meningitis to the rare meningitis due to dimorphic or filamentous fungi. *Cryptococcus, Candida, Aspergillus* and a series of molds can produce life-threatening CNS infections. These infections require immediate and precise diagnosis, as well as carefully selected management approaches to optimize outcomes.

Many of the etiologic agents of fungal meningitis may cause brain abscesses. *Candida* spp. had emerged as the most prevalent etiologic agents followed by *Aspergillus* spp., but also *Cryptococcus neoformans* and other fungi can be causative agents. In addition, many other fungi have also been reported to cause brain abscesses less frequently; these include *Scedosporium apiospermum, Paracoccidioides brasiliensis, Cladophialophora bantiana, Bipolaris hawaiiensis, Bipolaris spicifera, Exophiala dermatitidis, Ochroconis gallopava, Ramichloridium mackenziei* and *Curvularia pallescense*. Unfortunately, the diagnosis of fungal brain abscesses is often unexpected and many cases are not discovered until autopsy (Dotis *et al.*, 2007).

CNS fungal infections may present as a mass (brain abscess) typically in the course of aspergillosis or zygomycosis, or may primarily involve the meninges (meningitis), as can be observed in patients with candidiasis or cryptococcosis. *Cryptococcus neoformans* and the endemic fungi are the most common causes of CNS infections in immunocompetent patients, whereas *Candida*, *Aspergillus* species, and zygomycetes are among the most frequently cited causes of such infections in immunocompromised patients.

CNS fungal infections in hematological malignancies

In patients with hematological malignancies, opportunistic infections with *Candida*, cryptococcosis, or *Aspergillus* remain the most common infections affecting the CNS; however, opportunistic infections with less well-known fungi are becoming more common and must be considered in the differential diagnosis.

CNS aspergillosis is observed particularly in acute leukemia and allogeneic hemopoietic stem cell transplantation patients. Usually, aspergillosis is localized in the lungs and secondarily spreads to the brain. It develops only in a few cases as a solitary localization of CNS. In these conditions, diagnosis is very difficult because signs and symptoms can be completely nonspecific. Diagnosis can often be performed only through aggressive procedures such as stereotactic puncture. Members of Zygomycetes are the second most frequent cause of brain abscesses. CNS involvement is higher than in the course of invasive aspergillosis, and this fungal complication is also characterized by a high mortality rate. Studies demonstrated that only posaconazole and lipid formulations of amphotericin B present some possibility of success in the treatment of zygomycosis, but the pharmacologic approach should always be associated with surgery. Among molds, other agents such as *Fusarium* and *Scedosporium* may also be responsible for fungal abscess (Pagano *et al.*, 2005; Mattiuzzi *et al.*, 2005).

CNS fungal infections in transplant patients

Central nervous system (CNS) infections, account for 4-29% of CNS lesions in transplant recipients and are a significant post-transplant complication. Focal CNS infectious lesions or brain abscesses have been documented in 0.36-1% of the transplant recipients (Singh *et al.*, 2000). Mycelial fungi, particularly *Aspergillus*, are by far the most frequent etiologies of post-transplant brain abscesses. Most CNS complications occur early following orthotopic liver transplant but may be seen even after one year (al Hedaithy *et al.*, 1988; Bonham *et al.*, 1998; Bronster *et al.*, 2000).

Fungal brain abscess is an unusual but serious complication associated with solid organ and hematopoietic stem cell transplantation. One study reviewed cases of fungal brain abscess diagnosed in 1,620 adult patients who underwent allogeneic or autologous stem cell, liver, heart, lung, or renal transplantation. Seventeen cases of fungal brain abscess were identified that occurred a median of 140 days post-transplantation. Fungal brain abscess was more common among allogeneic stem cell transplant recipients. *Aspergillus* species were most commonly isolated, but unusual opportunistic molds were also identified. Multiple brain lesions were commonly seen on imaging studies. Although fungal brain abscess is an uncommon disease in this population, the outcome was poor, suggesting that early recognition of this disease might be helpful (Baddley *et al.*, 2002).

Another study assessed the autopsy findings of CNS infections in bone marrow transplant (BMT) recipients on 845 patients. 180 patients had autopsy with review of their medical records. Twenty-seven (15%) patients had brain parenchyma infection. Fungi were isolated in approximately 60% of the cases. Mean survival time was 153 days (0-1,264 days) and the majority of the patients died during the first 3 months after BMT (18 cases; 67%). *Aspergillus* sp. were the most prevalent fungi (30%), followed by Candida sp. infection (18%). There was one case of *Fusarium* sp. infection and two cases of unidentified fungus. All

patients with fungal infections had documented involvement at widespread sites. *Toxoplasma gondii* encephalitis was demonstrated in 8 patients (30%). Bacterial abscesses were responsible for approximately 11% of the findings. Eleven (41%) of the 27 patients died secondary to cerebral causes. These results demonstrated that infectious involvement of the CNS following BMT is a highly fatal event, caused mainly by fungi and *T. gondii*. Furthermore, they provide a likely guide to the possible causes of brain abscesses following BMT (de Medeiros *et al.*, 2000).

Infection represents the most frequent neurological complication in kidney transplant recipients. Acute meningitis usually caused by *Listeria monocytogenes*, sub-acute and chronic meningitis caused by *Cryptococcus neoformans*, focal brain infection caused by *Aspergillus fumigatus, Toxoplasma gondii* or *Nocardia asteroids*, and progressive dementia caused by polyoma J virus or other viruses are the most frequent types of neurological infections (Ponticelli *et al.*, 2005).

The epidemiological features of brain abscess formation after solid organ transplantation suggest 2 populations of patients exist that differ in timing, clinical setting, and response to therapy. For the chronically immunosuppressed outpatient, an established abscess should be empirically treated with sulfonamides until tissue diagnosis is confirmed. On the other hand, the acutely immunosuppressed post-transplant recipient, with defined risk factors, should receive full-dose therapy with amphotericin B and concomitantly had immunosuppression lowered (Selby *et al.*, 1997).

CNS Fungal infections in Acquired Immunodeficiency Syndrome (AIDS) patients

Opportunistic infections of the central nervous system (CNS) are common complications of advanced immunodeficiency in individuals with human immunodeficiency virus type 1 (HIV-1) infection. Neurological disease is the first manifestation of acquired immunodeficiency syndrome (AIDS) in 10% to 20% of symptomatic HIV-1 infection (Mamidi *et al.*, 2002).

The range of opportunistic infections occurring in association with HIV-1 is very broad. These infections develop most frequently in the background of marked immunosuppression. There is no part of the CNS that is immune to these complications. The concurrence of more than one infectious disease should always be considered (Wright *et al.*, 1997).

Histopathologic findings in the central nervous system in 100 autopsy cases of acquired immunodeficiency syndrome (AIDS), from the USA, gave evidence of a variety of opportunistic infections including CMV, toxoplasmosis, cryptococcosis and Candida (Rhodes, 1987). In a similar autopsy study from India, the spectrum of neuropathological brain lesions in HIV/AIDS cases between 1988 and mid-1996 at a tertiary level public hospital revealed opportunistic infections in 33 cases. These included toxoplasmosis (11 cases, 13%), tuberculosis (10 cases, 12%), cryptococcosis (seven cases, 8%), and cytomegalovirus infection

(six cases, 7%) (Lanjewar *et al.*,1998). In a study from Berlin, Germany, 200 patients who died with AIDS were examined retrospectively. A significant incidence of opportunistic infections was found particularly cerebral toxoplasmosis in 68 patients (34%). Cerebral cryptococcosis was found in three patients.

A high index of suspicion of intracranial fungal infection should exist for immunocompromised patients with intracranial lesions and diabetic patients with intracranial and rhinocerebral mass lesions (Dubey *et al.*, 2005).

Therapy of fungal central nervous system (CNS) infections is influenced by multiple factors including the pathogen and its drug susceptibility and by drug activity in the CNS, brain and spinal cord. Central nervous system drug levels are affected by the blood-brain barrier and the potential effect of efflux transporters that can modulate drug concentrations in the cerebrospinal fluid (CSF) and neural tissues. Early diagnosis, surgical decompression, and a complete course of promptly initiated antifungal therapy are associated with better prognosis.

Specific Fungal Infections

Aspergillosis

Aspergillus is an ubiquitous mold that can cause a wide variety of clinical syndromes ranging from mere colonization to fulminant invasive disease. Invasive aspergillosis is the most severe presentation of aspergillosis. The lung is usually the portal of entry, from which the pathogen may disseminate to almost any organ, often the brain and skin. The diagnosis remains a significant challenge. It is usually based on a combination of compatible clinical findings in a patient with risk-factors and isolation of the microorganism, radiological data, serological detection of antibodies or antigens, or histopathological evidence of invasion.

CNS aspergillosis is an uncommon infection, with high mortality, that most frequently occurs as an opportunistic fungal infection (Garcia *et al.*, 2006; Cho *et al.*, 2007). Craniocerebral aspergillosis can occur in immunocompetent hosts. In such hosts, it has three patterns of presentations that seem to correlate with clinical outcomes (Siddiqui *et al.*, 2004).

- Type 1: Intracerebral aspergillosis is associated with the worst clinical outcome.
- Type 2: Intracranial extradural aspergillosis remains intermediate outcome.
- Type 3: Cranial base and orbital aspergillosis has good recovery.

CNS aspergillosis may result from two different mechanisms most commonly through hematogenous dissemination of primary hematogenous aspergillosis and less often via direct extension from anatomically adjacent areas such as sinuses, ears, and orbits. In comparison with cerebrum, the cerebellum and brainstem are less commonly affected. The mechanism of invasiveness of

this organism remains unclear. It is probably caused by cellular or subcellular immunodeficiency. CNS aspergillosis is favored by working in agriculture, craft work, and by tropical climate (Murthy *et al.*, 2001; Sood *et al.*, 2007).

Risk factors for invasive aspergillosis include major or prolonged neutropenia, bone marrow or solid organ transplant (Torre-Cisneros *et al.*, 1993), AIDS (Mylonakis *et al.*, 2000; Vidal *et al.*, 2005), steroids and immunosuppressive therapy (Okamoto *et al.*, 1996; Stankovic *et al.*, 2006), idiopathic thrombocytopenic purpura (Tsai *et al.*, 2006), hematologic malignancies (Pagano *et al.*, 1996, 2005), hepatic failure, burns, intravenous drug abuse, diabetes mellitus, postoperative nosocomial infections complicating neurosurgical operations, and alcoholism (Torre-Cisneros *et al.*, 1993; Chandler *et al.*, 1997; Murthy *et al.*, 2001; Siddiqui *et al.*, 2004).

CNS aspergillosis is a life-threatening disease that carries a mortality rate approaching 100% in immunocompromised patients. Little is known about this serious infection in the pediatric population. CNS aspergillosis most commonly presents as single or multiple brain abscesses. While prematurity is the predominant underlying condition among infants, leukemia is the most frequent underlying disease in children. *Aspergillus fumigatus* is isolated from 75% of the cases. Children contracting CNS aspergillosis while undergoing systemic chemotherapy for leukemias represent a particularly unfortunate prognostic group (Middelhof *et al.*, 2005). CNS aspergillosis in infants and children predominantly presents as brain abscess(es) and has significantly better outcome compared to published adult data (Dotis *et al.*, 2007).

CNS aspergillosis is a relatively uncommon cause of brain expansile lesion in AIDS patients. In one study in which authors reported 6 new patients and reviewed 33 cases of AIDS who developed CNS aspergillosis. *Aspergillus* was found to most commonly involve the lungs, sinuses, ears, and orbits, while in one-fourth of the cases CNS was the only site of *Aspergillus* infection. The final diagnosis was made on autopsy in more than half these cases. CNS aspergillosis should be included in the differential diagnosis of HIV-infected patients who present with nonspecific neurologic symptoms and signs. In HIV-infected individuals it occurs more often as a result of direct extension from the sinuses, orbits, and ears than through hematogenous spread from the lungs (Mylonakis *et al.*, 2000; Vidal *et al.*, 2005).

In addition to the expected post-transplantation and hematological malignancy cases, other risk groups identified included those with chronic asthma and steroid use, acquired immunodeficiency syndrome, thermal burn, hepatic failure, and postoperative infection. Unusual cases manifested with basilar meningitis, myelitis, proptosis caused by sino-orbital disease, or epidural and subdural *Aspergillus* abscesses. The extent of gross neuropathologic disease ranged from subtle abscesses to massive hemorrhagic necrosis causing herniation and death. In addition to the expected hemorrhagic necrosis, extensive hemorrhage, focal purulent meningitis, and subtle bland infarctions were also seen. Distinctive microscopic findings encountered included one case with

numerous meningeal granulomas and multinucleated giant cells and four cases showing the Splendore-Hoeppli phenomenon (Kleinschmidt-DeMasters *et al.*, 2002).

Aspergillosis of the CNS should be considered patients with clinical features of headache, multiple cranial nerve palsies and alteration of consciousness accompanied by sinusitis, especially in elderly and diabetic patients. It remains a catastrophic opportunistic infection in spite of the current intensive and aggressive treatment (Dotis *et al.*, 2007). *Aspergillus flavus* has been reported to be the most frequently isolated species from cultures of invasive aspergillosis of nasal and paranasal origins (Alrajhi *et al.*, 2001). *Aspergillus fumigatus* is more frequently implicated as a cause of invasive aspergillosis in immuno-compromised patients (Siddiqui *et al*, 2004; Sood *et al.*, 2007).

Aspergillus fumigatus is the most common human pathogen in the genus *Aspergillus*. Maxillary sinusitis of dental origin or the lungs are the most common sites of primary *Aspergillus* infection. Infection reaches the brain directly from the nasal sinuses via vascular channels or is blood borne from the lungs and gastrointestinal tract. Single or multiple abscess formation with blood vessel invasion leading to thrombosis is a characteristic feature of Aspergillosis on neuropathologic examination. Aspergillosis should be considered in cases manifesting with acute onset of focal neurologic deficits resulting from a suspected vascular or space-occupying lesion especially in immunocompro-mised hosts. It can be diagnosed on direct examination of smear and culture; however, the diagnosis of aspergillosis of the CNS is difficult. Diagnosis of an intracranial mass lesion is best confirmed with a computed tomography or magnetic resonance imaging of the head with or without intravenous contrast. Aggressive neurosurgical intervention for surgical removal of *Aspergillus* abscesses, granulomas, and focally infracted brain; correction of underlying risk factors; Amphotericin B combined with flucytosine and treatment of the source of infection should form the mainstay of the management (Murthy *et al.*, 2000; Nadkarni *et al.*, 2005).

Cerebral aspergillosis presents three principal neuroimaging findings: areas consistent with infarction; ring lesions consistent with abscess formation following infarction; and dural or vascular infiltration originating from paranasal sinusitis or orbital infiltration. Recognition of these three patterns of aspergillosis in immunosuppressed patients may lead to more effective diagnosis and treatment planning (Ashdown *et al.*, 1994; DeLone *et al.*,1999; Yamada *et al.*, 2002; Siddiqui *et al.*, 2006; Tempkin *et al.*, 2006; Gabelmann *et al.*, 2007).

A preoperative diagnosis of aspergillosis is very difficult, but a perioperative squash smear/frozen section can identify the pathology (Alapatt *et al.*, 2006). Recent studies have found that the galactomannan antigen enzyme-linked immunosorbent assay and Polymerase chain reaction (PCR) test may be useful for early diagnosis (Musher *et al.*, 2004).

Conventional antifungal agents like amphotericin-B (Schwartz *et al.*, 2007) and itraconazole (Saulsbury *et al.*, 2001) are almost ineffective in cerebral aspergillosis. Newer azoles have been shown to penetrate the blood, cerebrospinal fluid barrier and achieve effective fungicidal concentrations. These newer azoles such as Variconazole may change the outlook of this fatal condition (Kerkmann *et al.*, 1994; Schwartz *et al.*, 2003, 2005; Wandroo *et al.*, 2006).

Antifungal medications prove ineffective for treating CNS aspergillosis in patients immunocompromised because of their chemotherapy regimens. In contrast, withholding chemotherapy to reverse immunosuppression, thereby improving the efficacy of antifungal medications, allows for progression of the primary leukemic disease. There is crucial role of stereotactic neurosurgery for the intelligent treatment of immunocompromised children suspected of harboring a CNS aspergilloma and abscesses. The goal for successful treatment in these patients should be gross-total resection of the abscess, its wall, and its capsule (Middelhof *et al.*, 2005). Post transplant studies have also shown poor penetration of amphotericin B into the brain and cerebrospinal fluid. One way to achieve therapeutic levels of the agent near the abscess is through the direct introduction of the agent into the abscess site via an indwelling catheter (Camarata *et al.*, 1992).

Treatment methods for aspergillosis include surgery, amphotericin B, lipid-associated amphotericin B, itraconazole, and newer antifungal agents, such as voriconazole or caspofungin. In general, treatment of invasive aspergillosis with antifungal agents has a high failure rate.

Although the best approach regarding the use of antifungal agents has not yet been determined, the most generally accepted approach is to administer the highest possible dose of amphotericin B. Recent published data show that voriconazole is highly effective compared with amphotericin B for the treatment of invasive *Aspergillus* infection (Dubey *et al.*, 2005).

Zygomycosis/Mucormycosis

Zygomycosis is a unifying term that encompasses various diseases caused by fungi of the class Zygomycetes. These are ubiquitous saprophytic organisms found on bread, soil, air, and decaying vegetation, especially in tropical countries (Gartenberg *et al.*,1978; Sundaram *et al.*, 2005).

The human pathogens in this class belong to the orders Mucorales and Entomophthorales, and the corresponding diseases are Mucormycosis and Entomophthoromycosis respectively (Chandler *et al.*, 1997b). These are non-septate fungi. Pathogenic fungi in the family Mucorales include *Apophysomyces* (Garcia-Covarrubias *et al.*, 2001; Schtz *et al.*, 2006; Liang *et al.*, 2006), *Cunninghamella* (Brennan *et al.*, 1983), *Saksenaea* (Gonis *et al.*, 1997), *Rhizopus* (Riefler *et al.*, 1991; Hofman *et al.*, 1993; Attapattu, 1995), *Absidia* (Eucker *et al.*, 2000), *Mucor, Syncephalastrum, Rhizomucor*, and *Mortierella*. Entomophthorales include *Basidiobolus*, and *Conidiobolus* (Chandler *et al.*, 1997b).

There are distinct clinical and pathologic differences between Mucormycosis and Entomophthoromycosis. Mucormycosis is a sporadic disease worldwide occurring mainly as an opportunistic infection in patients with acidosis, and immunosuppression. On the other hand, Entomophthoromycosis is not an opportunistic infection and is predominantly seen in Africa, Southeast Asia, and South America. It results from percutaneous implantation of the fungus (Chandler *et al.*, 1997b).

Zygomycosis is an opportunistic infection that may be localized to the site of entry such as sinuses, lungs, and skin, or can be disseminated. The infection usually occurs in patients with underlying disorders such as diabetes mellitus with ketoacidosis but may occur in otherwise healthy individuals (Fairley *et al.*, 2000; Sharma *et al.*, 2001; Sridhara *et al.*, 2005). Other predisposing conditions are seen in approximately 38% of cases, including: immunosuppressive therapy, leukemia, lymphoma, burns (Rabin *et al.*, 1961), trauma (Cocanour CS *et al.*, 1992), desferrioxamine therapy (Kaneko *et al.*, 1991; Boelaert *et al.*, 1993; Wu *et al.*, 2006), liver cirrhosis (Hofman *et al.*, 1993; Abbas *et al.*, 2007), aplastic anemia (Srensen *et al*, 2006), myelodysplastic syndrome (Kubota *et al.*, 2003), renal failure (Ray *et al.*, 2002), AIDS (Hejny *et al.*, 2001), intravenous drug users (Yoshiura *et al.*, 2001), leukemia, Non-Hodgkin lymphoma, renal transplant, systemic necrotizing vasculitis, gastroenteritis, glomerulonephritis, and hemodialysis (Berenguer *et al.*, 1990; Chandler *et al.*, 1997b; Sundaram *et al.*, 2005).

Rhino-orbital mucormycosis (ROM) is uncommon in patients with AIDS. When ROM occurs, a careful search for an underlying metabolic derangement such as neutropenia is required (Hejny *et al.*, 2001). Also in patients with hematological diseases, the infection most frequently occurs in neutropenic cases (Eucker *et al.*, 2001). Rhinocerebral mucormycosis (RCM) infrequently occurs in the pediatric population, and when it involves thrombosis of an internal carotid artery (Simmons *et al.*, 2005), it has been almost uniformly fatal (Pillsbury *et al.*, 1977; Meyers *et al.*, 1979; Kaneko *et al.*, 1991; Ray *et al.*, 2002; Kubota *et al.*, 2003; Khor *et al.*, 2003; Srensen *et al*, 2006 ; Mohindra *et al.*, 2007; Abbas *et al.*, 2007).

Rhino-orbital-cerebral mucormycosis (ROCM) is an acute, fulminant, often fatal, fungal infection. The infection progresses rapidly and causes necrosis and infarction of bony structures in and near the nasal cavities. *Rhizopus* accounts for most cases of ROCM. The rhinocerebral form of the disease, which comprises nearly one half of recently reported cases, is most often found in uncontrolled diabetics or profoundly dehydrated children. Carotid artery occlusion and cavernous sinus thrombosis are potential complications of RCM (Smith *et al.*, 1986; Johnson *et al.*, 1988; Quattrocolo *et al.*, 1990; Inamasu *et al.*, 2000; Delbrouck *et al.*, 2004; Gelston *et al.*, 2007). In patients who had both ROCM and diabetes (Bhansali *et al.*, 2004; Simmons *et al.*, 2005), ROCM was the presenting manifestation in one fourth of the patients.

The disease is characterized by fungal hyphal invasion of blood vessels resulting in thrombosis and infarction of the nasal, paranasal sinus, orbital, and

cerebral tissues. ROCM may have seasonal incidence peaking in the fall and early winter (Talmi *et al.*, 2002). Phycomycosis is the preferred terminology to define a fungal disease that may be devastating and fatal. A C.T. scan is very helpful for establishing orbital and intracranial extension. When intracranial involvement is present, the prognosis is dismal (Maniglia *et al.*, 1982; Mnif *et al.*, 2005).

Chronic presentations of rhinocerebral mucormycosis (CRM) have also been described. In this form of infection, the disease course is indolent and slowly progressive, often occurring over weeks to months. CRM occurs predominantly in patients with diabetes and ketoacidosis. The most common presenting features of CRM are ophthalmologic and include ptosis, proptosis, visual loss, and ophthalmoplegia. The incidence of internal carotid artery and cavernous sinus thrombosis is higher in CRM patients than in those with the acute disease, although the overall survival rate for CRM patients is 83%. CRM is clinically distinct from chronic Entomophthorales infection (Harril *et al.*, 1996; Rumboldt *et al.*, 2002; Bhansali *et al.*, 2004).

The following factors are related to a lower survival rate:

(1) Delayed diagnosis and treatment.

(2) Hemiparesis or hemiplegia.

(3) Bilateral sinus involvement.

(4) Leukemia.

(5) Renal disease.

(6) Treatment with deferoxamine.

The association of facial necrosis with a poor prognosis fell just short of statistical significance, but appears clinically important (Yohai *et al.*, 1994). The term zygomycosis is preferred to mucormycosis when the diagnosis is made on tissue sections without culture confirmation.

Definitive diagnosis is made by demonstration of fungal hyphae in tissue specimens. Preoperative cytology is an effective technique to establish a diagnosis of mucormycosis and obviates the need for a preoperative biopsy. Frozen section is a specific and sensitive method to make a quick initial diagnosis of RCM (Deshpande *et al.*, 2000; Hofman *et al.*, 2003; Safar *et al.*, 2005).

The mainstay of treatment is aggressive surgical debridement of infected tissue and administration of amphotericin B. ROCM has a mortality rate of 40-50%; 70% of survivors are left with residual defects. Early diagnosis and treatment are imperative in the successful management of patients afflicted with this devastating sight- and life-threatening disease (Warwar *et al.*, 1998).

Rhinocerebral mucormycosis should be suspected in diabetic patients with ketoacidosis who do not respond to antibiotic therapy. Treatment begins with correction of the underlying condition and is followed by surgery. Because

zygomycosis is intrinsically resistant to most antifungal agents, the preferred medical therapy is amphotericin B.

Hyperbaric oxygen suppresses fungal growth *in vitro* and has theoretical value in treating mucormycosis because it reduces the tissue hypoxia and acidosis that accompany vascular invasion by the fungus. Adjunctive hyperbaric oxygen appears to be a promising clinical modality for the treatment of rhinocerebral mucormycosis and warrants further investigation (Ferry *et al.*, 1983; Ferguson *et al.*, 1988; Yohai *et al.*, 1994; Peterson *et al.*,1997; Talmi *et al.*, 2002; Nithyanandam *et al.*, 2003; Dubey *et al.*, 2005; Sundaram *et al.*, 2006).

Early diagnosis is critical in the prevention of intracranial extension of the infection, which is the cause of death in 80% of cases. Therefore, a high index of clinical suspicion is essential in immuno-compromised or diabetic patients with acute sinus infection. A team approach to management is recommended for early surgical debridement, correction of diabetic ketoacidosis, and systemic antifungal agents. Timely medical-surgical treatment proves extremely important for prognosis (Ochi *et al.*, 1988; Blzquez *et al.*, 1996; Raj *et al.*, 1998; Alobid *et al.*, 2001; Kofteridis *et al.*, 2003; Barron *et al.*, 2005; Safar *et al.*, 2005).

Phaeohyphomycosis

Phaeohyphomycosis comprises a heterogenous group of fungal infections caused by a wide variety of dematiaceous (phaeoid or naturally pigmented) fungi that develop as black molds in culture and as dark walled moniliform fungal elements in tissue. The etiologic agents of this mycosis include more than 80 recognized genera and species of opportunistic fungi. The fungi contain melanin in their cell walls, imparting a characteristic dark color to their conidia and hyphae. It may also play an important role in the pathogenesis of these fungi. Melanin is a known virulence factor in other fungi. It remains unclear why these fungi preferentially affect the CNS. An intriguing possibility is that melanin itself may be responsible for this CNS localization (Kantarcioglu *et al.*, 2004). The infection is characterized by the basic development of dark colored filamentous hyphae in the invaded tissue. These fungi exist in hyphal to yeast forms in the tissue and have been variously referred to as dematiaceous, phaeoid, or darkly pigmented. Other synonyms include chromomycosis, cerebral dematiomycosis, and phaeosporotrichosis (Brandt *et al.*, 2003; Revankar *et al.*, 2004, 2006, 2007).

Many species of dematiaceous fungi are associated with human disease though only a few are responsible for most cases. There are two major clinical forms: subcutaneous form called phaeomycotic cyst, and a systemic form with involvement of CNS.

The subcutaneous form is the commonest form and is caused by either dematiaceous pigmented or eumycotic non-pigmented fungi present in soil, wood, and decaying plant material. These fungi gain access to the tissue via a wooden splinter or thorn. The most frequently encountered fungi include *Exophiala jeanselmei, Phialophora parasitica, P. richardsiae, Wangiella*

dermatitidis, Bipolaris spicifera, Alternaria alternata and *Curvularia (*Sheikh *et al.*, 2007).

In the systemic form involving CNS, the route of infection is usually through inhalation of fungal elements that are growing as a mycelium in the environment.

The most frequently encountered agents include *Cladophialophora bantiana (C. bantiana C. trichoides*) (Binford *et al.,* 1952; Dixon *et al.,*1989; Deb *et al.,* 2005; Roche *et al.*, 2005; Tunuguntla *et al.*, 2005) which is the most common species affecting humans followed by *Ramichloridium mackenziei.* The latter is seen exclusively in patients from the Middle East. Other agents include *Bipolaris hawaiiensis, B. spicifera* (Adam *et al.,* 1986), *Dactylaria constricta var gallopava* (Vukmir *et al.*, 1994), *Fonsacaea pedrosi* (al Hedaithy *et al.,* 1988; Nbrega *et al.,* 2003), and *F. monomorpha (*Surash *et al.,* 2005; Takei *et al.*, 2007), W. *dermatitidis* (Chang *et al.,* 2000), and Curvularia (Carter *et al.*, 2004) among others (Yoshimori *et al.*, 1982; Chandler *et al.*, 1997c; Filizzola *et al.,* 2003; Revankar *et al.,* 2004; Takei *et al.*, 2007).

There are three main clinical types of CNS infections by pigmented fungi (Kantarcioglu *et al.,* 2004):

1- Primary cerebral infection where the first symptoms and localizations are of a neurologic nature. This is predominantly caused by fungi of the order Chaetothyriales, comprising the black yeasts and related species such as Exophiala, Cladophialophora, and Ramichloridium.

2- Secondary cerebral infections extending from adjacent tissues such as sinuses. This is usually caused by grass-inhabiting fungi "Pleosporales" comprising Bipolaris, Dissitimurus, and Exserohilum.

3- Extra-cerebral infections with cells being present in cerebrospinal fluid (CSF). It is most commonly caused by essentially non-pigmented fungus Pseudallescheria, a member *of Microascales.*

4- Meningitis had been practically absent in pigmented fungi but was observed repeatedly in recent years.

Cladophialophora bantiana (Deb *et al.,* 2005; Roche *et al.,* 2005; Tunuguntla *et al.,* 2005), Exophiala dermatidis (Tintelnot *et al.,* 1991), Ramichloridium mackenziei (Kanj *et al.,* 2001), and Ochroconis gallopavum (Sides *et al.,* 1991; Wang *et al.,* 2003) are considered to have a strong predilection for the nervous system and thus truly neurotropic pigmented fungi. Bipolaris spicifera Bipolaris hawaiiensis, and Curvularia pallescens are only rarely involved in CNS infections (Chandler *et al.,* 1997c).

Cladophialophora bantiana has several older names in literature including Cladosporium trichoides (Binford *et al.,* 1952), Cladosporium bantianum (Roche *et al.,* 2005; Tunuguntla *et al.,* 2005), and Xylohypha bantiana (Dixon *et al.,* 1989; Lee *et al.,* 2003). It was first isolated by Binford *et al.* (1952) in a patient with cerebral hyphomycosis (Binford *et al.,* 1952). It is almost exclusively

pathogenic to humans and neurotropic. Although reported worldwide there is a general preference for warmer climates with higher humidity. Most commonly seen in young male immunocompetent patients, however approximately 40% of cases have an underlying factor such as organ transplant (Salama *et al.,* 1997; Silveira *et al.,* 2003), or drug abuse (Walz *et al.,* 1997). Some patients may present with multiple brain abscesses (Tunuguntla *et al.,* 2005) or in children (Trinh *et al.,* 2003) posing a diagnostic challenge. Occasional cases are reported that clinically mimic cerebral aspergillosis (Lee *et al.,* 2003).

Ramichloridium mackenziei has only been reported from Middle East region reflecting a limited geographic range for this organism. It is the only neurotropic fungus reported from that area, and in Saudi Arabia it is one of the most common causes of CNS fungal infection. Cerebral phaeohyphomycosis by *R. mackenziei* was first reported in an immunocompetent host (ur Rahman *et al.*, 1988). Several cases are reported, most involving the cerebrum, with one third of the patients being immunocompetent while the other two third are immunocompromised or had been subjected to a surgical procedure (Kantarcioglu *et al.,* 1999; Kanj *et al.,* 2001). Several patients have underlying disorders such as Diabetes (Amr *et al.*, 2007), renal failure (Podnos *et al.*, 1999), chronic myelomonocytic leukemia (Kanj *et al.,* 2001), systemic lupus erythematosus (Amr *et al.*, 2007), myelofibrosis with Hodgkin's lymphoma (Sutton *et al.,* 1998), and renal transplant recipient on immunosuppressive therapy (Campbell *et al.*, 1993). Mortality is 100% without surgery, but 65% when the lesion is excised (Jamjoom *et al.*, 1995; Kanj *et al.,* 2001; Khan *et al.*, 2002; Kashgari *et al.*, 2002).

Rare cases of CNS infection by other pigmented fungi are reported. Examples include *B. spicifera* most probably causing secondary infection to preceding acoustic neuroma, and *Curvularia clavata* cerebritis following sinusitis (Ebright *et al.*, 1999; Latham *et al.*, 2000). Infection caused by Bipolaris and *Aspergillus* show many clinical and pathologic similarities despite the lack of taxonomic relationship between these fungi. Both can cause disseminated disease in immunocompromised patients that is characterized by tissue necrosis and vascular invasion. Both cause CNS disease, osteomyelitis, and sinusitis, and are associated with allergic bronchopulmonary disease (Adam *et al.*, 1986).

Other organisms that may lead to cerebral phaeohyphomycosis include *Chaetomium* (Abbott *et al.,* 1995; Thomas *et al.*, 1999), *Scopulariopsis* (Guppy *et al.*, 1998; Thomas *et al.*, 1999; Baddley *et al.,* 2000; Teixeira *et al.,* 2003) , and *Scedosporium apiospermum*, the anamorph or the asexual forms of *Pseudallescharia boydii* . Disseminated mycosis due to Scedosporium species (*S. apiospermum and S. prolificans*) have been reported in patients with underlying hematologic malignancies such as Burkitt lymphoma (Nenoff *et al.*, 1996), acute lymphoblastic and myelogenous leukemias (Marin *et al.,* 1991; Alvarez *et al.*, 1995; Berenguer *et al.*, 1997), chronic granulomatous disease (Bhat *et al.*, 2007), transplant recipients (Husain *et al.*, 2005), AIDS patients (Nenoff *et al.*, 1996), and occasionally in non-immunocompromised hosts especially children (Barbaric *et al.*, 2001). Several cases of CNS mycosis caused by *Scedosporium*

apiospermum Pseudallescharia species have been reported after drowning or near-drowning incidences in polluted waters of streams or ponds with still water (Dworzack *et al.*, 1989; Rchel *et al.*, 1995; Kowacs *et al.*, 2004; Buzina *et al.*, 2006; Panichpisal *et al.*, 2006; Mursch *et al.*, 2006).

Cerebral phaeohyphomycosis almost always evokes a granulomatous response with many giant cells (Kanj *et al.*, 2001; Deb *et al.*, 2005). The overall survival rate in patients with this infection is 28-35%. Although most cases involve cerebrum, a few cases are reported to involve cerebellum and ventricles. In 25% of cases the patients present with symptoms of meningitis.

The diagnosis of phaeohyphomycosis can be difficult because dematiaceous fungi are commonly soil inhabitants and are often considered contaminants when identified in culture. Identification of these fungi is based mostly upon morphology. Important structures include septation and germination, annellides, phialides, differentiation of conidiophores and conidial hilum, adelophialides, and phialides (Dixon *et al.*, 1991). Early recognition is highly significant and the requirements for successful therapy are complete resection supplemented with adequate antifungal therapy. The clinical outcome of cerebral and other deep-seated forms of phaeohyphomycosis is dismal. An aggressive therapeutic approach is required given the high mortality rate associated with these infections (Kantarcioglu *et al.*, 2004).

Cryptococcosis

Cryptococcosis, known in the past as torulosis, is systemic mycosis, described for the first time over 100 years ago in 1894, caused by an encapsulated basidiomycetous, yeast-like fungus, *Cryptococcus neoformans*. There are two variants and four major serotypes of this organism, namely *Cryptococcus neoformans*, var. *neoformans* (Serotypes A and D) and *Cryptococcus neoformans* var. *gatti* (Serotypes B and C) (Mitchell *et al.*, 1995). *C. neoformans* was found by Kwon-Chung to represent the asexual or anamorphic form of a heterothallic basidiomycete, which she named Filobasidiella neoformans (Kwon-Chung *et al.*, 1976), *C. neoformans* var. *neoformans* is an ubiquitous saprophyte of soil and avian habitats, particularly those heavily contaminated with pigeon excreta (Halde *et al.*, 1966; McDonough *et al.*,1966). C. neoformans var. gatti had been associated with eucalyptus trees which grow in tropical areas (Ellis *et al.*, 1990). There are 16 other species of the genus *Cryptococcus*, but they are not pathogenic to man. A thick polysaccharide capsule is characteristic of *C. neoformans*, although on rare occasions, minimal or no capsular material is found. This thick capsule is the basis of the India ink test on cerebrospinal fluid for the detection of this organism (Fujita *et al.*, 1980).

Although the fungus is pathogenic for those with competent immunity, it is more often encountered as an opportunistic infectious agent and is the most common cause of fungal meningitis. It has become increasingly prevalent in immunocompromised patients. Given the rarity of this organism infecting an

immunocompetent host it is considered a unique manifestation in healthy individuals. Factors that predispose to opportunistic cryptococcosis include AIDS (Dismukes *et al.*, 1988; Chuck *et al.,* 1989; Dixon *et al.,* 1991; Collazos, 2003), prolonged treatment with corticosteroids (Wilson *et al.*, 1970; Schulman *et al.*, 1988), lupus erythematosus (Speller *et al.*, 1977; Zimmermann *et al.,* 1992;), sarcoidosis (Botha *et al.,* 1999; Ross *et al.,* 2002; Kanaly *et al.*, 2007), diabetes mellitus, organ transplantation (Tilney *et al.*, 1982; Husain *et al.,* 2001; Akamatsu *et al.*, 2005), malignancy, particularly Hodgkins disease (Korfel *et al.*, 1998), and other conditions known to impair cell-mediated immunity. Invasive cryptococcal infection is rare in healthy immunocompetent individuals. It is an extremely neurotropic organism.

Clinically, cryptococcosis occurs in two basic forms, pulmonary cryptococcosis and cerebro-meningeal cryptococcosis. The former is considered as the initial primary site of infection being the site of portal of entry through inhalation of the yeast forms of the organism. The latter is acquired by hematogenous or lymphatic dissemination from a primary pulmonary focus. Careful assessment of the CNS is required in cryptococcosis to rule out occult meningitis. It is not a contagious disease that is acquired by inhaling aerosolized cells of C. neoformans that are produced in the environment.

The majority of the patients (70-90%) present with the usual signs and symptoms of subacute meningitis or meningoencephalitis, with headache being the most common symptom, but patients may also present with meningeal signs, confusion, seizures, blurred vision, lethargy, personality changes and memory loss; and rarely, focal deficits. Unlike bacterial meningitis, nuchal rigidity may be mild or absent. Lumbar puncture is a useful initial diagnostic test that shows increased CSF pressure and may show mild-to-moderate leukocytosis, decreased glucose levels, and elevated protein levels. The India ink test is more specific and helps in demonstrating the fungus. The level of antigen titer corresponds to the severity of disease (Fujita *et al.*, 1980).

Besides the immune status of the patient, the size of the inoculum is considered to be an important factor in determining the pathogenesis of this disease. Infection usually starts as meningitis. Parenchymal involvement is seen as cryptococcomas (also known as toruloma), dilated Virchow-Robin spaces, or enhancing cortical nodules. It is believed that the meningeal infection along the base of the skull may involve the adjacent brain parenchyma, giving rise to cryptococcomas or may extend along the Virchow-Robin spaces. These are perivascular spaces seen accompanying the lenticulo-striate, perforating branches of the middle cerebral arteries in the basal ganglia. As the infection spreads along the Virchow-Robin spaces, the perivascular spaces may dilate with mucoid gelatinous material produced by the capsule of the organism. These cysts have, therefore, also been called "gelatinous pseudocysts." In patients with AIDS and other profound immunodeficiencies, proliferating cryptococci may produce large extracellular aggregates or "yeast lakes" that efface the normal architecture of an organ. In otherwise healthy immunocompetent patients, *C. neoformans* usually

elicits a mixed suppurative and granulomatous inflammatory cellular reaction or a purely granulomatous reaction with varying degree of necrosis (Chandler *et al.*, 1997d; Dubey *et al.*, 2005).

Imaging tests may provide useful diagnostic information (although the findings are not pathognomonic and other infectious processes may simulate cryptococcal infection in the brain). A communicating hydrocephalus may occur because of the acute meningeal exudate and also may occur late in the course of the infection because of meningeal adhesions (Cornell *et al.*, 1982).Gelatinous pseudocysts are seen as multiple CSF-equivalent round or oval cysts in the basal ganglia, thalami, midbrain, cerebellum, and the periventricular regions (Popovich *et al.,* 1990). On MR imaging, these are seen as multiple hypointense T1 and hyperintense T2 lesions. Demonstration of clusters of these cysts in the basal ganglia and thalami is fairly specific and strongly suggestive of this infection. It has been recommended that longer duration of treatment and combination therapy, with multiple antifungal agents, might be needed not only in HIV-infected and resistant cases but also in infected immunocompetent patients. In conclusion, the appearance of a Cryptococcus central nervous system infection in an immunocompetent patient may be different from that commonly encountered in the immunocompromised. Cryptococcosis should be considered when multiple cystic lesions are noted in the basal ganglia, even in an immunocompetent patient. Marked ring enhancement of the basal ganglia lesions may be seen; this enhancement probably represents the patient's ability to mount an immune response, which would be uncommon in an immunocompromised patient. Marked surrounding edema may also be a feature of this infection (Chandler *et al.* , 1997d ; Saag *et al.*, 2000; Pruitt, 2003, 2004; Saigal *et al.*, 2005).

Rare cases of cryptococcal meningitis developing encephalopathy, secondary to Amphotericin therapy are reported in the literature. Even more rare is the development of encephalopathy in cryptococcal meningitis patients unrelated to Amphotericin treatment, possibly as an immune response to the organism (Liu *et al.*, 1995; Wilcox *et al.,* 2007).

Candidiasis

Candida organisms are readily detectable as harmless commensals in the flora of the mouth, pharynx, vagina, and skin. In normal hosts these organisms are usually not invasive. In immunocompromised patients they are capable of invading and causing severe disease. Candida species are the fifth most common primary bloodstream organisms and the seventh most common cause of nosocomial infections. Cerebral candidiasis is the result of hematogenous dissemination. The gastrointestinal tract is the most common source of systemic infection in patients with hematological malignancies and neutropenia, as a result they have a high incidence of hepatic and splenic involvement. Patients with adequate neutrophil counts who develop disseminated candidiasis after abdominal surgery or from intravenous catheter-induced phlebitis (Burgert *et al.*, 1995), tend to have myocardial, pulmonary, renal, ocular, and cerebral

involvement. Cerebral involvement by Candida is usually not recognized until autopsy because it is often masked by the patient's underlying primary disease. Furthermore the lesions tend to be small and focal, and are scattered randomly throughout the cerebral hemispheres, the basal ganglia, and cerebellum. Characteristically, many of these lesions are located near the middle cerebral artery. The lesions include both intraparenchymal abscesses and small vessel thrombosis formed by pseudohyphae with associated microinfarcts surrounding areas of vasculitis. Histologically the usual response to Candida is an acute inflammatory reaction with neutrophils predominating and causing microabscesses. In neutropenic patients the inflammatory reaction is replaced by coagulative necrosis. The inflammatory reaction depends on the immunologic status of the individual patient (Chandler *et al.*, 1997e). Brain abscesses are rare, with the reported overall incidence in patients with disseminated candidiasis ranging from below 1% to 2%. Various autopsy series have put the incidence at 7% to 17%. Hosts who are at highest risk for cerebral candidiasis are newborns and patients who are neutropenic. Meningitis is the most usual form of Candida infection of the central nervous system. It may present in one of the three forms: chronic Candida meningitis, Candida meningitis in patients with acquired immune deficiency syndrome (AIDS), and Candida meningitis in patients who underwent neurosurgical procedures.

Chronic Candida meningitis is an uncommon manifestation of candidiasis. In a report of an elderly woman affected with this disease, with a review of 17 similar cases, it was found that these patients had a clinical course characterized by a headache that progressed over a period of weeks or even months and is associated with confusion, vomiting, stupor, hydrocephalus, and coma. Nuchal rigidity was present in 60% of cases. Known risk factors were present in 72% of cases. Only 17% of CSF smears were positive and only 44% of initial CSF cultures grew Candida species. The overall mortality rate was 53%, and in those treated and followed up it was 33% (Voice *et al.*, 1994). In another study of 3 patients, all were intravenous drug abusers, who developed chronic neutrophilic meningitis, the interval between the onset of the disease and the diagnosis was long, ranging from 4 to 12 months. All three patients developed hydrocephalus. Treatment with amphotericin, 5-flucytosine and fluconazole resulted in clinical improvement and sterilization of CSF in all three patients (del Pozo *et al.*, 1998).

Candida meningitis in AIDS patients has a subacute course that usually runs for fewer than 4 weeks and is predominantly related to intravenous drug use. In AIDS patients, having low CD4 counts is not a risk factor for invasive or disseminated candidiasis, but most affected patients have additional risk factors, such as drug-induced neutropenia. In a series of 14 cases of candidal meningitis in HIV-infected patients, the median CD4 cell count was 135/mm^3. Headache and fever, in the absence of focal neurologic signs, were the predominant clinical features. In spite of treatment with amphotericin B for a median of 51 days, the overall mortality was 31% (Casado *et al.*, 1997) Risk factors for Candida meningitis in neurosurgery patients include long-term use of ventriculo-

peritoneal shunts and broad-spectrum antibiotics, and recent neurosurgery. Therapy consists of a combination of shunt removal and antifungal agents (Geers *et al.*, 1999; Montero *et al.*, 2000).

Candidal meningitis had been reported in children with cancer. In one report of 12 children, all had leukemia, it was found that antibiotic therapy, duration of fever, profound neutropenia and use of total parental nutrition were significantly associated with candidal meningitis when compared with matched control subjects (McCullers *et al.*, 2000).

Disseminated or systemic candidiasis is a major cause of morbidity and mortality in premature infants with very low birth weight (VLBW). CNS involvement can complicate such systemic infection. In a review of 106 cases of systemic candidiasis in neonates, 23 had candidal meningitis. The median birth weight was 820 grams, and the median age at the onset of the disease was 8 days. CSF findings were variable and non-specific. Candida was isolated in 17 neonates (74%). Nine patients (35%) died, three of them were not treated with anti-fungal therapy, and diagnosed at the post-mortem examination. Those who survived were treated with amphotericin B alone (17 patients) or with flucytosine (4 patients) (Fernandez *et al.*, 2000).

Histoplasmosis

Histoplasma capsulatum is a systemic fungal infection contracted by inhaling airborne infectious conidia originating in soil. The primary sources of most infections are avian and chiropteran (bats) habitats that favor growth of this thermally dimorphic fungus in soil that is enriched with feces. Primary pulmonary lesions in *H. capsulatum* are usually subclinical and heal without antifungal therapy, but they may calcify and be discovered at chest X-rays. The remaining 5-10% of the infections are symptomatic and fall into three broad categories – acute pulmonary, chronic pulmonary, and disseminated forms. Disseminated infection is the most severe and life-threatening form, usually occurring in immunocompromised patients, infants and children (Rivera *et al.*, 1992), and the elderly. It frequently complicates organ transplant recipients (Livas *et al.*, 1995), AIDS (Vullo *et al.*, 1997; Knapp *et al.*, 1999; Manning *et al.*,2006; Azizirad *et al.*, 2007), hematological malignancies, and chemotherapy, all conditions characterized by deficient cell-mediated immunity (Chandler *et al.*, 1997f). The infection can occur in immunocompetent hosts (Hott *et al.*, 2003).

Hematogenous dissemination results in slow but progressive spread of infection into various organs. CNS involvement is clinically recognized in 5%–10% of cases of progressive disseminated histoplasmosis (PDH). Clinical syndromes include subacute or chronic meningitis, focal brain or spinal cord lesions, stroke syndromes, and encephalitis. CNS involvement may be a manifestation of widely disseminated disease or an isolated illness, occurring as the initial manifestation of PDH or relapse at a "privileged" body site poorly penetrated by antifungal therapy. Occasionally solitary lesions, called "intracranial histoplasmomas," can clinically mimic brain tumors. Clinicians

should maintain a high index of suspicion in patients who are from endemic areas or with an underlying predisposing condition (Klein *et al.*, 1999; Paphitou *et al.*, 2002).

Often, the diagnosis is not suspected, leading to chronic, untreated infection and, in some cases, to placement of ventricular shunt for normal-pressure hydrocephalus. The mortality rate in the disseminated form is as high as 80% without antifungal therapy. The diagnosis should be suspected in patients with chronic meningitis or parenchymal lesions, for which the results of tests for other causes are negative, particularly if the patient has been to areas where histoplasmosis is endemic. Once diagnosis is suspected, a panel of tests is needed to achieve the highest sensitivity for diagnosis. The optimal management is uncertain, but, given the high rates of failure of initial therapy (~20%) and relapse in the next few years (~40%), an aggressive approach is recommended (Chandler *et al.*, 1997f; Assi *et al.*, 2007). Rare cases of cerebral histoplasmosis have been reported, presenting 13 years after the primary infection (Bamberger *et al.*, 1999).

Coccidioidomycosis

Coccidioidomycosis is an infectious disease that is acquired by inhaling fungal conidia of *Coccidioides immitis*. *C. immitis* exists in soil in limited areas where there are hot summers and low rainfalls, making the soil sandy and alkaline with abundant ash, such as San Joaquin Valley of southern California as well as Arizona and New Mexico and a few Central American countries. It is a diphasic and multimorphic organism. Although *C. immitis* infections are usually asymptomatic or result in mild, flu-like illness, disseminated disease may occur in 1% of cases. The infection may range from silent infection, which is the most common presentation, to progressive infection and death. While extrapulmonary disease usually involves the skin, CNS, bones, and joints, coccidioidomycosis can causes a wide variety of lesions and, like syphilis, is called a "great mimicker or imitator" (Galgiani, 1993; Chandler *et al.*, 1997g; Chiller *et al.*, 2003).

Dissemination is reported to be more often in African Americans, Filipinos, and Asians than Caucasians. Other predisposing factors that help in dissemination are immunosuppression, such as in AIDS, hematologic malignancies, organ transplant, blood group B, and onset in second and third trimester of pregnancy. There are four basic clinical forms – symptomatic or asymptomatic pulmonary, primary cutaneous, residual pulmonary, and disseminated infection. Only about 5% of the patients with clinically apparent pulmonary infection have disseminated disease. Histologically there is a predominantly suppurative reaction to recently released endospores and a granulomatous reaction to maturing spherules. The inflammatory response and rate of replication are influenced by the patient's immune status and by underlying disease (Chandler *et al.*, 1997g).

Coccidioidal meningitis affects between 200-300 persons annually within the endemic area of the United States, with much larger numbers expected in epidemic years (Williams, 2007). *C. immitis* involvement of CNS usually begins in the form of subacute granulomatous meningitis with occasional patients developing brain abscesses (Kleinschmidt-DeMasters *et al.*, 2000; Davis *et al.*, 2005). The central nervous system infection results when *Coccidioides immitis* disseminates from a primary lung infection via a fungemia to reach the meninges (Davis *et al.*, 2005). Few case reports of brain abscess due to *C. immitis* had been reported (Mendel *et al.*, 1994; Bauelos *et al.*, 1996). One report from California documented ten cases of vasculitis of CNS, with one associated with encephalitis, all due to *C. immitis* (Williams *et al.*, 1992). Another report described the pathological findings in 9 cases including one patient with AIDS (Mischel *et al.*, 1995). There was a spectrum of neuropathological change ranges from meningitis to meningoencephalitis and meningomyelitis with extensive parenchymal destruction, sometimes as a result of an associated endarteritis obliterans (Chandler *et al.*, 1997g; Chiller *et al.*, 2003). Early in the disease course coccidioidal meningitis may show areas of focal enhancement in the basal cisterns, which may progress to diffuse disease. Pathologically the areas of enhancement represent focal collections of the organism. Deep infarcts and communicating hydrocephalus are associated findings (Erly *et al.*, 1999).

Diagnosis may be challenging because *C. immitis* is isolated from cerebrospinal fluid in less than 50% of patients. A cerebrospinal fluid complement fixation test for IgG antibody to *C. immitis* has high sensitivity and specificity (Davis *et al.*, 2005).

Blastomycosis

Blastomycosis is a systemic infection caused by *Blastomyces dermatitidis*, a thermally dimorphic fungus. The route of entry is often through inhalation of aerosolized infectious conidia growing in soil with the primary focus being in the lungs. Primary cutaneous infection is rare, usually caused by accidental inoculation of the fungus into the skin. This form is an occupational hazard for pathologists and microbiologists and also has occurred following dog bites. Systemic blastomycosis begins in the lungs where it either remains confined there or disseminates hematogenously to other organs including the brain. Untreated systemic infections are often severe, progressive, and fatal with the mortality rate being more than 90%. Like most other fungal infection, blastomycosis is most often seen in immunodeficient hosts. In immuno-compromised patients the infection tends to disseminate more often, in particular to the central nervous system (Chandler *et al.*, 1997h). Although blastomycosis of the CNS occurs in approximately 4% of patients with blastomycosis, patients can present with recurrent CNS blastomycosis in extremely rare occasions (Chowfin *et al.*, 2000; Wu *et al.*, 2005). Rare cases are reported in hosts working in fields after Hurricane Katrina (Szeder *et al.*, 2007).

Histologically the fungi are often numerous, extracellular, and scattered throughout the lesion with suppurative inflammatory reaction and formation of abscesses. In immunocompromised patients and rapidly progressive lesions the fungi proliferate in great numbers forming "yeast lakes" with only minimal inflammatory response. Amphotericin B, alone or sometimes in combination with other antifungal agents (Panicker *et al.*, 2006; Borgia *et al.*, 2006), is the drug of choice for severe life-threatening disease. Voriconazole was reported to be successful in treatment of cerebral blastomycosis as well (Bakleh *et al.*, 2005; Borgia *et al.*, 2006). Surgical excision of localized lesions may be a valuable adjunct to the antifungal therapy (Benzel *et al.*, 1986; Chandler *et al.*, 1997h; Friedman *et al.*, 2000).

Paracoccidioidomycosis

Paracoccidiodomycosis (PCM) is a systemic infection caused by *Paracoccidiodes brasiliensis* that is recovered from soil. PCM is an infectious disease caused by a dimorphic fungus, endemic in subtropical areas of Central and South America (de Almeida *et al.*, 2004, 2005). Primary lesions of PCM are usually seen in young hosts as a self-limiting pulmonary infection with only rare progression. Years after infection, reactivation of a primary quiescent lesion may become progressive chronic PCM of the lung, with or without involvement of other organs. Acute or subacute disseminated PCM predominantly occurs in children, and young adults. Any organ can be involved; however the mononuclear phagocytic system is most severely affected. Most symptoms are related to the gastrointestinal tract, osteoarticular system, with lymphadenopathy and abdominal symptoms. Mucous membrane lesions are uncommon.

The involvement of CNS is higher than previously thought and two clinical presentations have been reported, meningitis and pseudotumor. The diagnosis of CNS involvement is difficult and it is important to consider neuroPCM in the differential diagnosis of brain and spinal cord lesions in endemic areas of PCM as clinical suspicion is a key point to achieve the correct diagnosis. CNS lesions commonly involve brain hemispheres and thalamus, followed by cerebellum, brainstem, and spinal cord. Epilepsy is one of the commonest neurologic presentations (de Almeida *et al.*, 2004). Neuroimaging diagnosis showed a predominance of multiple round lesions with ring enhancement following contrast medium injection (de Almeida *et al.*, 2004; Elias *et al.*, 2005; Paniago *et al.*, 2007). Chronic disseminated PCM are most commonly seen in young adult males. Pulmonary lesions are present in almost all patients. Extrapulmonary lesions are mostly localized to mucous membranes, lymph nodes and skin. Less frequently other organs are affected such as the central nervous system, genitals, liver, and spleen. Histologically the most common host response is granulomatous with or without associated suppurative reaction. *P brasiliensis* can be recognized in H&E (Hematoxylin and Eosin stain) stained sections however characteristic features are best seen by GMS stain (Gomori Methenamine silver stain). Diagnosis of PCM is based on identification of the classic tissue forms of

the fungus complemented by cultures. The antifungal agent of choice is often Ketoconazole with surgical excision of localized lesions as a valuable adjunct to the antifungal therapy (Chandler FW *et al.*, 1997i).

Sporotrichosis

Sporotrichosis is a chronic cutaneous or systemic mycosis caused by *Sporothrix schenckii.* It is caused by accidental inoculation of conidia growing in soil and plant material such as thorns, barbs, and splinters. Often the infection remains localized to skin, subcutaneous tissue, and the contagious lymphatics. Rarely dissemination occurs to other organs including meninges, joints, bones, lungs, and genitourinary system. Disseminated sporotrichosis may occur in immunodeficient individuals but meningitis remains a rare complication (Hardman *et al.*, 2005; Silva-Vergara *et al.*, 2005; Vilela *et al.*, 2007).

On very rare occasions, infection is acquired through inhalation of the fungal conidia causing primary pulmonary infection which also may disseminate subsequently. Hosts with profound immunodeficiency, AIDS (Penn *et al.*, 1992; Donabedian *et al.*, 1994; Hardman *et al.*, 2005; Silva-Vergara *et al.*, 2005; Vilela *et al.*, 2007) serious underlying diseases, and chronic alcoholics, are at the greatest risk of disseminated infection (Chandler *et al.*, 1997i).

Systemic sporotrichosis is often localized to a single organ with only occasional dissemination via hematogenous route to multiple organs. Diagnosis is usually difficult, requiring isolation of the organism from the CSF or skin so appropriate treatment can be promptly initiated (Hardman *et al.*, 2005).

Histologically the infection is characterized by florid pseudoepitheliomatous hyperplasia, ulceration, and intraepidermal microabscesses with only a few fungi present in tissue sections stained with H&E; however the fungi are clearly demonstrated by GMS stain. The infection elicits both pyogenic and granulomatous reaction. The fungi may be surrounded by Splendore-Hoeppli material that is refractile and intensely eosinophilic, forming an asteroid body. The asteroid body is almost always seen in microabscesses or in the suppurative centers of the granulomas. Asteroid bodies are not seen in many cases of sporotrichosis and when present are not considered pathognomonic for this infection, as similar reactions can be seen around bacteria such as actinomycosis, botryomycosis, and mycetoma, parasitic ova, foreign material, and may even occur in association with other fungi such as coccidiodomycosis, aspergillosis, candidiasis, and entomophthoromycosis (Chandler *et al.*, 1997j; Silva-Vergara *et al.*, 2005).

Penicillinoses

The most common cause of systemic penicillinosis is *Penicillium marneffei* (Chandler *et al.*, 1997k; Noritomi *et al.*, 2005). It was formerly considered as an extremely rare infection, however, currently it is one of the common opportunistic infections in AIDS patients, in particular in Southeast Asia (Kurup

et al., 1999). It is the only *Penicillium* species which is dimorphic and can cause systemic mycosis in humans. It is endemic in Southeast Asia and China (Supparatpinyo *et al.*, 1994). *Penicillium* species other than *P.marneffei* cause only superficial or allergic disease with only rare cases of invasive disease occurrence.

Conclusion

It can be concluded that fungal infections of the CNS have been observed with more frequency in the last two decades. Clinicians, including: physicians, surgeons, radiologists, and pathologists,and mycologists are all challenged when it comes to the diagnosis and treatment of these unusual infections. A high index of suspicion should be kept in mind, in particular when an immunocompetent patient presents with neurological deficits.

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CHAPTER - 6

OTOMYCOSIS IN TURKEY: A REVIEW

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Introduction

Otomycosis is the fungal infection of the ear. Although it usually implies fungal infection of the external ear canal, the middle ear may be involved in case of a perforated tympanic membrane, and the mastoid cavity may be affected if an open cavity mastoid surgery is performed previously. Otomycosis may rarely involve inner ear in immunocompromized hosts (Haruna *et al.*, 1994). In this case, fungal infection is invasive. Invasive fungal infection usually accompanies immunosuppression due to cancer chemotherapy. Diabetes mellitus may be another predisposing factor for invasive fungal disease. In such a case, hospitalization of the patient and systemic treatment is necessary.

Otomycosis causes significant morbidity due to its bothersome symptoms such as itching, aural discharge, hearing loss, and otalgia. Swimming, previous history of tympanic membrane perforation or ear surgery, and presence of dermatomycoses such as tinea pedis must alert physician for otomycosis especially in the presence of itching of the ear.

Otomycosis may be challenging for both patient and the physician since long term treatment and follow up is needed, and still the recurrence rate is high. The treatment of otomycosis may be costly due to long treatment and follow up periods. Improper diagnosis and treatment may increase direct costs of the disease such as physician fee and medicine costs as well as indirect costs such as absenteeism related to hospital visits. The exact cost of otomycosis is not known in Turkey or in the United States. However, it is reported that the ototopical market in the United States is approximately 7.5 million annual prescriptions with total sales of $310 million (Rosenfeld *et al.*, 2006).

Otomycosis shows a worldwide distribution and is particularly frequent in hot and humid regions (Than *et al.*, 1980; Paulose *et al.*, 1989; Yehia *et al.*, 1990; Asci *et al.*, 1996; Kaur *et al.*, 2000; Ozcan *et al.*, 2003a). The prevalence of otomycosis is as high as 54% in hot and humid regions (Than *et al.*, 1980) whereas the prevalence decreases to 9% in temperate climates (Mugliston and O'Donoghue, 1985).

Otomycosis has been reported from almost every region in Turkey (Akiner *et al.*, 1991; Durmaz *et al.*, 1991; Erkan and Soyuer, 1991; Sivrel *et al.*, 1992; Asci *et al.*, 1996; Gurer *et al.*, 1998; Koc *et al.*, 1998; Ozcan *et al.*, 2000; Degerli *et al.*, 2002; Ozcan *et al.*, 2003a; Uslu *et al.*, 2005). The majority of the patients (65.5%) in central Anatolia admit to hospital in summer and early autumn when the weather is hot in this region (Ozcan *et al.*, 2003a).

Otomycosis is a disease of adulthood (Than *et al.*, 1980; Paulose *et al.*, 1989; Yehia *et al.*, 1990; Erkan and Soyuer, 1991; Asci *et al.*, 1996; Kaur *et al.*, 2000; Ozcan *et al.*, 2003a) although the children may also be affected (Jackman *et al.*, 2005; Ho *et al.*, 2006). The disease is unilateral in approximately 90% of the patients without any preponderance of the right or left sides (Paulose *et al.*,1989; Yehia *et al.*, 1990; Kaur *et al.*, 2000; Ozcan *et al.*, 2003a; Ho *et al.*, 2006). Some authors report that it is more frequent in females (Yehia *et al.*,1990; Ozcan *et al.*, 2003a) while some others claim that it is more common in males (Kaur *et al.*, 2000; Ho *et al.*, 2006).

Risk factors for otomycosis

Wearing turban or other clothes on head has been reported as a risk factor for otomycosis in Bahrain, Turkey and India (Paulose *et al.* 1989; Ozcan *et al.*, 2003a; Kumar, 2005). In our opinion, turban impedes evaporation of the sweat in the head region, and the humidity in the external ear canal increases. This creates a suitable environment for the fungal growth. In Turkey, only women wear turban because of religious issues, and this may be the reason for more frequent occurrence of otomycosis in women.

Swimming is reported as a risk factor for otomycosis (Bryant, 1948; Paulose *et al.*, 1989; Wang *et al.*, 2005). Approximately 28% of the patients in our study had history of swimming prior to development of otomycosis (Ozcan *et al.*, 2003a). Majority of them had a bath in the warm pool of a health spa. Otomycosis following swimming in warm pool of a health spa may be due to inhabitance of some fungi in these pools.

Perforation of the tympanic membrane and/or previous ear surgery have been reported as important risk factors for otomycosis (Falser, 1984; Paulose *et al.* 1989; Vennewald *et al.*, 2003; Ho *et al.*, 2006). The rate of tympanic membrane perforation was 3.5% in Central Anatolia and it was not regarded as a major risk factor for otomycosis (Ozcan *et al.*, 2003a). However, tympanic membrane perforation was reported as a major predisposing factor for otomycosis in a study performed in Southern Turkey (Ozcan *et al.*, 2000).

Presence of dermatomycoses may be a factor for the unresponsiveness to treatment. The prevalence of dermatomycoses in patients with otomycosis was 36.5% in Turkey (Ozcan *et al.*, 2003) and 51% in India (Kumar, 2005). The prevalence of dermatomycoses was reported as 5.7% in general population (Perea *et al.*, 2000). Similarly, the prevalence of dermatomycoses was 5% in our control group (Ozcan *et al.*, 2003a). The prevalence of dermatomycoses seems significantly higher in patients with otomycosis.

In Turkey, 50% of the patients that were refractory to otomycosis treatment had dermatomycoses such as tinea pedis (Ozcan *et al.*, 2003a). The same pathogenic fungi were isolated from dermatomycoses and otomycosis in nearly half of these patients (Ozcan *et al.*, 2003b). It was interesting that *Aspergillus species* were the most common shared pathogens isolated from both dermatomycoses and otomycosis (Ozcan *et al.*, 2003b). *Aspergilli* are unusual pathogens for dermatomycoses in the general population. Identification of *Aspergilli* in dermatomycoses suggests a digital contamination of the toes.

Topical antibiotics have been frequently used for treatment of the bacterial infections of the external ear canal in the last decades. Use of topical antibiotics has been reported as a predisposing factor for otomycosis by some authors (Jackman *et al.*, 2005), while some others disagreed (Stern and Lucente, 1988; Paulose *et al.*, 1989).

There is no agreement whether the presence of earwax in the ear canal predisposes individuals to otomycosis. Both absence (Paulose *et al.*, 1989) and presence (Stern and Lucente, 1988; Kaur *et al.*, 2000) of earwax in the external ear canal have been accused as predisposing factors for otomycosis. Some authors suggested that cleaning external ear canal with matchsticks or cotton bud swabs was a predisposing factor (Kaur *et al.*, 2000; Ozcan *et al.*, 2000).

Seborrheic dermatitis on face is reported as another predisposing factor for otomycosis (Erkan and Soyuer, 1991). Other proposed predisposing factors are immunosuppression, radiation therapy, cancer chemotherapy and prolonged treatment with systemic antibiotics (Falser, 1984).

Symptoms

Presence of itching in external ear canal must raise a high index of suspicion for otomycosis since it is the most frequent symptom. Itching is present in more than 90% of the patients (Yehia *et al.*, 1990; Erkan and Soyuer, 1991; Ozcan *et al.*, 2000; Ozcan *et al.*, 2003a; Pradhan *et al.*, 2003). Other common symptoms are otalgia, hearing loss, tinnitus and aural discharge (Ozcan *et al.*, 2003a). Otalgia is usually seen together with aural discharge. Hearing loss and tinnitus are usually due to obstruction of the external ear canal by aural discharge or the hyphae.

The onset of otomycosis is usually abrupt. In some patients, fungal infection may supervene on bacterial external otitis and in that case itching may follow

pain. In immunocompromised host, systemic signs of infection such as fever may be present.

Diagnosis

Clinical diagnosis of otomycosis requires a high index of suspicion. The history must include symptoms, their duration, and the topical or systemic medications used before the patient admitted the hospital. If the patient used any topical preparation, the nature of it must be questioned since it may be confused with the ear discharge on otoscopic examination.

The patient must be questioned for the presence of the predisposing factors that are mentioned above. Presence of chronic systemic disorders such as diabetes must be questioned. Swimming, previous history of tympanic membrane perforation or ear surgery, and presence of dermatomycoses such as tinea pedis must alert physician for otomycosis especially if the patient complains of itching. Patient must be questioned whether he/she had otomycosis previously. It must be kept in mind that recurrences are common in otomycosis.

After history, a careful otoscopic examination is necessary. Presence of mycelia in the external ear canal is a characteristic finding for otomycosis (Fig. 6.1), however, absence does not rule out the diagnosis. The mycelia can be seen in the external ear canal in 23-62% of the cases (Erkan and Soyuer, 1991; Ozcan *et al.*, 2003a).

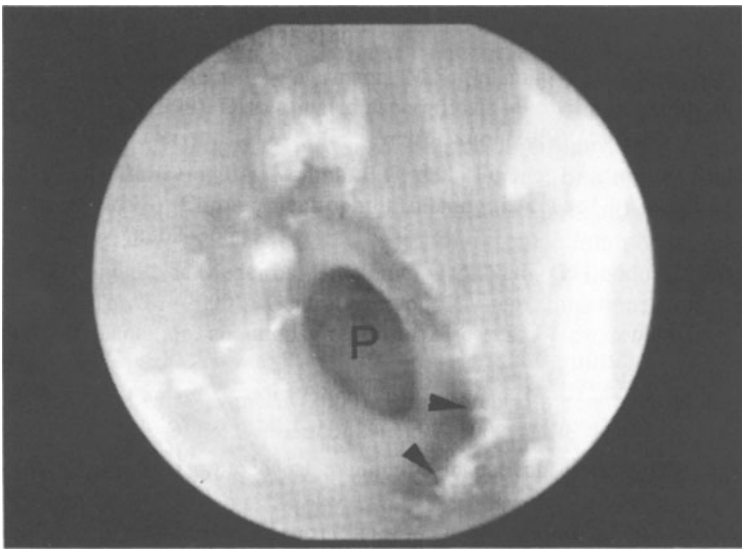

Fig. 6.1. Mycelia in the external ear canal. The arrowheads indicate the hyphae. P = Perforation of the tympanic membrane.

Presence of blotting paper like material consisting of fungal elements and epithelial debris in the external ear canal is also suggestive of otomycosis

especially in the patient with the complaint of itching. Purulent aural discharge may also be seen in the external ear canal.

Signs of inflammation such as edema and erythema may be seen in the ear canal skin. The skin can usually be seen only after cleansing the fungal elements and debris in the ear canal. The tympanic membrane may show the signs of inflammation such as myringitis and maceration. A perforation may be seen in the tympanic membrane. In such a case, the patient must be questioned for the history of an eardrum perforation, and his/her medical records must be examined if possible. As mentioned before, a perforated tympanic membrane may predispose to otomycosis. However, otomycosis may also cause perforation of the tympanic membrane (Hurst, 2001; Ho *et al.*, 2006). In case of previous canal wall down mastoidectomy, mycelia may be seen in the mastoid cavity (Fig. 6.2).

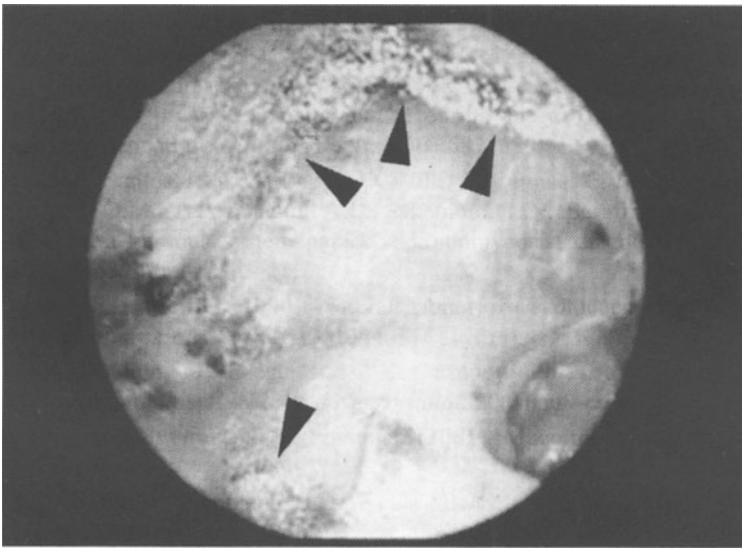

Fig. 6.2. Mycelia in an open mastoid cavity. The patient previously had a radical mastoidectomy. The arrowheads indicate mycelia.

The patient must be questioned for the presence of dermatomycoses and he/she must be examined for them. If present, dermatomycoses must be treated simultaneously with otomycosis to prevent recurrences.

In immunocompromised hosts, systemic signs of infection such as fever may be present. In this case, fungal infection may be invasive. Invasive fungal infection usually accompanies immunosuppression due to cancer chemotherapy. It can also be seen in diabetics. Mucor species have been demonstrated in cochlea and auditory nerve in autopsy in a patient died because of leukemia, and those findings are indicative of invasive fungal infection (Haruna *et al.*, 1994). Invasive fungal infection of the ear is an emergency and hospitalization is necessary. Systemic antifungals must be administered. Surgical debridement may be needed to control the infection.

Identification of causative fungi must be a part of the diagnosis. The mycelia/debris must be removed from the external ear canal and examined for fungal elements under microscope. Ten per cent potassium hydroxide solution is used for this purpose. Fungal cultures must be performed for identification of the causative fungus. Sabouraud dextrose agar is the most commonly used medium. The physician must not wait for the culture results to begin the treatment since it may take two weeks for fungi to grow in cultures.

Fungi causing otomycosis

Aspergillus and *Candida species* are the most common pathogenic fungi that cause otomycosis in the world as well as in Turkey. It is suggested that *Aspergillus species* were the most common fungal isolates in hot and humid countries whereas in temperate regions there was a preponderance of *Candida* (Stern and Lucente, 1988). The most common pathogenic fungi that were isolated from the patients with otomycosis in the world are presented in Table 6.1.

Table 6.1. The most common isolates from the ears with otomycosis in the world.

Country	Causal fungus	%
Mosul, Iraq (Yehia *et al.*, 1990)	*Aspergillus niger*	70.9
Ahwaz, Iran (Mahmoudabadi, 2006)	*Aspergillus niger*	50
AlKhobar, Saudi Arabia (Bassiouny *et al.*, 1986)	*Aspergillus niger*	36.5
Bahrain (Paulose *et al.* 1989)	*Aspergillus niger*	54.4
Enugu, Nigeria (Mgbor and Gugnani, 2001)	*Aspergillus niger*	43.1
Ramatswa, Botswana (van Hasselt and Gudde, 2004)	*Aspergillus fumigatus*	52.9
Kathmandu, Nepal (Pradhan *et al.*, 2003)	*Aspergillus flavus*	32.5
New Delhi, India (Kaur *et al.*, 2000)	*Aspergillus fumigatus*	41.1
Dehradun, India (Kumar, 2005)	*Aspergillus niger*	52.4
Madrid, Spain (del Palacio *et al.*, 2002)	*Aspergillus niger*	42.5
Lodz, Poland (Kurnatowski and Filipiak, 2001)	*Candida parapsilosis*	29.3
Dresden, Germany (Vennewald *et al.*, 2003)	*Aspergillus niger*	20
	Candida parapsilosis	20
New York, USA (Jackman *et al.*, 2005)	*Candida albicans*	42
Innsbruck, Austria (Falser, 1984)	*Candida albicans*	32

Turkey is situated in the Mediterranean region and its south and west coasts are quite hot and humid especially in the summer. Central Anatolia is also hot although it is relatively less humid compared to the coastal regions. Major pathogenic fungi for otomycosis are *Aspergillus species* in most of Turkey. The major pathogenic fungi isolated from the patients with otomycosis in different regions of Turkey are shown in Table 6.2.

Table 6.2. The major fungi isolated from otomycosis in different regions of Turkey.

Place/Country	Causal fungus	%
Ankara (Ozcan *et al.*, 2003a)	*Aspergillus niger*	44.8
Ankara (Akiner *et al.*, 1991)	*Aspergillus niger*	75
Elazig (Asci *et al.*, 1996)	*Aspergillus niger*	64
Manisa (Degerli *et al.*, 2002)	*Aspergillus niger*	50.5
Mersin (Ozcan *et al.*, 2000)	*Aspergillus niger*	26.6
Istanbul (Gurer *et al.*, 1998)	*Aspergillus niger*	43.8
Izmir (Sivrel *et al.*, 1992)	*Aspergillus niger*	69.7
Erzurum (Uslu *et al.* 2005)	*Aspergillus fumigatus*	18.4
Kayseri (Erkan and Soyuer, 1991)	*Penicillum spp.*	29.5

Both fungi and bacteria were isolated from ears with otomycosis in some studies (Paulose *et al.*, 1989; Yehia *et al.*, 1990; Asci *et al.*, 1996; Ozcan *et al.*, 2000; Ozcan *et al.*, 2003a). The most common bacteria isolated were *Streptococcus epidermidis* (Ozcan *et al.*, 2003a), *Staphylococcus aureus* (Yehia *et al.*, 1990; Asci *et al.*, 1996) and *Pseudomonas aureus* (Paulose *et al.*, 1989). *Staphylococcus aureus* and *Enterobacteriaceae* had equal prevalences in one study (Ozcan *et al.*, 2000). The importance of mixed fungal and bacterial infections is not known, however, presence of a mixed infection may indicate resistance to therapy. In our series, 80% of the patients who were refractory to initial therapy had mixed infections (Ozcan *et al.*, 2003a).

Treatment

Topical therapy is usually preferred in otomycosis. Systemic treatment is rarely needed and must be reserved for the patients that are unresponsive to topical therapy and for the immunsuppressed patients.

Frequent cleansing of external ear canal is the mainstay of therapy, and early reports on the treatment of otomycosis emphasize its importance (Bryant, 1948). Cleansing fungi and debris from the external ear canal removes irritative fungal material out of the external ear canal and enables topical preparations to contact with the canal skin. Cleansing may be performed with cotton tipped applicators or by suction. I prefer suction cleansing. Cleansing of the external ear canal must be performed carefully to avoid any damage to inflamed skin and the eardrum. Using an operating microscope may be helpful in this stage of the treatment.

Topical preparations used in the treatment of otomycosis may be divided into three groups: (i) Antiseptics, (ii) Antifungals, (iii) Others. A variety of antiseptics and antifungals have been used for the treatment of otomycosis, however, there is no consensus on which agent to be preferred. Some authors prefer antiseptic agents only, some use antifungals only and some use a combination of them. The antiseptics, antifungals and the other agents used in the treatment of otomycosis are listed in Table 6.3.

Table 6.3. Topical agents used in the treatment of otomycosis.

ANTISEPTICS
Acetic acid 2% (Tom, 2000)
Acetic acid 2% plus hydrocortisone 1% (Erkan *et al.*, 1993)
Thiomerosal 1% (Stern and Lucente, 1988)
Aluminum acetate 2% (Stern and Lucente, 1988; Ho *et al.*, 2006)
Ethyl alcohol 95% (Stern and Lucente, 1988)
Boric acid 4% in alcohol (Ozcan *et al.*, 2000; Ozcan *et al.*, 2003a; Ozcan *et al.*, 2003b; Karaarslan *et al.*, 2005)
Merbromin (Mgbor and Gugnani, 2001; Gutierrez *et al.*, 2005)
Castellani solution (Vennewald *et al.*, 2003)
ANTIFUNGALS
Clotrimazole (Bassiouny *et al.*, 1986; Paulose *et al.* 1989; Mgbor and Gugnani, 2001; Ozcan *et al.*, 2000; Perea *et al.*, 2000 ; Vennewald *et al.*, 2003)
Econazole nitrate (Bassiouny *et al.*, 1986)
Miconazole nitrate (Bassiouny *et al.*, 1986; Stern and Lucente, 1988; Gurer *et al.*, 1998)
Ciclopox olamine (Bassiouny *et al.*, 1986 ; Gutierrez *et al.*, 2005)
Nystatin (Stern and Lucente, 1988; Gurer *et al.*, 1998)
Amphotericin B (Stern and Lucente, 1988)
Tolnaftate (Stern and Lucente, 1988)
Natamycin (Stern and Lucente, 1988; Kurnatowski and Filipiak, 2001)
Cliaquinol plus hydrocortisone (Mgbor and Gugnani, 2001)
Fluconazole (Kurnatowski and Filipiak, 2001)
m-cresyl acetate (Ho *et al.*, 2006)
Ketokonazole (Ho *et al.*, 2006)
Tioconazole (Ozcan *et al.*, 2003a; Ozcan *et al.*, 2003b)
Bifonazole (Falser, 1984)
OTHERS
Polymyxin B plus neomycin plus hydrocortisone (Stern and Lucente, 1988; Stern *et al.*, 1988)

To culture fungus causing otomycosis for identification is time consuming, and probably not possible in some institutions. In addition to that, bacterial and fungal mixed infections are not uncommon. Those factors may constitute the basis for the use of antiseptics in the treatment of otomycosis. In addition to that, antiseptics are cheaper when compared to the antifungals. Although all of the antiseptics listed in Table 6.3 were reported as clinically effective, *in vitro* effectiveness of merbromin (Stern *et al.*, 1988) and boric acid 4% in alcohol (Karaarslan *et al.*, 2005) have been shown against *Aspergillus* and *Candida species* that were isolated from the ears with otomycosis. In addition to their

efficacy on causative fungi, the antiseptics aid the physician during the cleansing of the external ear canal.

Topical antifungals are marketed in the forms of powder, cream or lotion. They have been primarily manufactured for the treatment of dermatomycoses. In most countries including Turkey, there are no otic preparations for antifungals, and dermal preparations are used in otomycosis. The antifungals listed in Table 6.3 have been used with varying success in the treatment of otomycosis in the world and in Turkey.

In our institution we use boric acid 4% in alcohol as eardrops twice daily in the initial therapy of otomycosis. Careful suction cleansing of the external ear canal is an important part of the therapy and is performed every other day in the first week of the treatment. The patient is instructed to obstruct his/her ear canal while showering. This treatment regimen is the preferred treatment modality in other institutions in Turkey (Erkan *et al.*, 1993; Ozcan *et al.*, 2000). If the patient is resistant to therapy after two weeks, we apply topical 1% tioconazole cream to the external ear canal once a day. The patient continues to use topical alcohol boric eardrops. This treatment regimen resulted in resolution of otomycosis in 100% of our patients in 4 weeks.

Tioconazole is an imidazole derivative and is effective against most fungi including *Aspergillus* and *Candida species in vitro* (Jevons *et al.*, 1979; Fromtling, 1988). Tioconazole is also effective *in vivo* (Ozcan *et al.*, 2003a).

Use of a topical agent is a major concern in every patient with a perforated tympanic membrane in terms of toxicity to inner ear or an adverse effect on hearing (i.e. ototoxicity). The same is true for patients that have an open cavity mastoidectomy. Some agents commonly used in the treatment of otomycosis have been shown to be ototoxic (Marsh and Tom, 1989; Tom, 2000), however, ototoxicity of most of the agents have not been studied yet. The physician must be very careful in terms of ototoxicity while treating otomycosis in patients with perforated tympanic membranes.

Conclusion

Otomycosis is frequent in hot and humid regions of the world and it requires appropriate treatment. Identification of the causative fungi is necessary, however, the treatment must begin just after the clinical diagnosis. *Aspergillus* and *Candida species* are the most common pathogenic fungi that cause otomycosis in the world as well as in Turkey. Mixed infection (fungi + bacteria) is not uncommon especially in stubborn cases.

The treatment of otomycosis is usually topical. Frequent cleansing of the external ear canal is an important component of the therapy. Dermatomycoses, if present, must be treated simultaneously to avoid re-contamination and frequent relapses.

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CHAPTER - 7

CURRENT TRENDS IN FUNGAL RHINOSINUSITIS

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Introduction

Rhinosinusitis is a significant health problem which can be result in a major financial cost to society (Ray *et al.*, 1999). The condition represents a complicated and multifaceted group of diseases, with fungal aetiology representing one of its distinct entities. It is therefore extremely important to be able to accurately diagnose each entity and assess the underlying factors in order to determine appropriate therapeutic measures.

The incidence of mycotic infections and the number and diversity of pathogenic fungi have increased dramatically in recent years in both immunocompetent and immunocompromised individuals. Fungal rhinosinusitis is one of the fungal diseases that are increasing in incidence and are often difficult to diagnose and treat (Ragab and Clement, 2007).

This chapter considers some recent developments in the classification, terminology, diagnosis and management of different fungal forms of rhinosinusitis.

Classification and clinical definition of rhinosinusitis

Rhinosinusitis can be defined as any inflammation of the nasal and para-nasal sinus mucosa that results in signs and symptoms. Most classification systems of rhinosinusitis, however, are based on the duration of the symptoms and/or the specific sinus involved.

In 1997, the International Rhinosinusitis Advisory Board (Lanza and Kennedy, 1997) published the clinical classification of rhinosinusitis in adults. In this scheme the clinical symptoms and signs were divided into major and minor

criteria (see Table 7.1). Acute rhinosinusitis (ARS) was defined as a condition that lasted for less than 12 weeks, and chronic rhinosinusitis (CRS) was defined as where symptoms had been present for 12 or more weeks, and patients had a combination of two or more major factors, or one major factor and two minor factors (Lanza and Kennedy, 1997).

Table 7.1. Criteria associated with diagnosis of rhinosinusitis

Major factors	Minor factors
Facial pain/pressure*	Headache
	Fever (all non acute)
Nasal obstruction/Blockage	Halitosis
Nasal discharge, purulence, or discoloured postnasal drainage	Fatigue
Hyposmia/anosmia	Dental pain
Purulence in nasal cavity in examination	Cough
	Ear pain/pressure/fullness

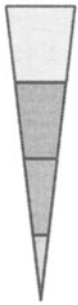
*Facial pain/pressure alone does not constitute a suggestive history for rhinosinusitis in the absence of another major nasal symptom or sign. Modified from (Lanza and Kennedy, 1997).

A more recent European position paper on rhinosinusitis and nasal polyps endorsed these criteria, but also recommended confirmation by nasal endoscopy or computed tomography scan. Endoscopic signs include the presence of polyps, mucopurulant discharge from the middle meatus or edematous and mucosal obstruction primarily in the middle meatus. The computed tomography changes are mucosal swelling or fluid levels within the ostiomeatal complex or sinuses. The European position paper proposed that the severity of sinus disease was scored on a visual analogue scale for the symptoms, and endoscopic or computed tomography scores for the clinical findings (Fokkens *et al.*, 2005).

Classification and forms of fungal rhinosinusitis

Most infections of the sinuses affect not only the paranasal sinuses but also the nose. The American Academy of Otolaryngology's Task force group suggested that the term rhinosinusitis was a more accurate description of the disease process than the term sinusitis. Six forms of fungal rhinosinusitis disorders are currently recognized (Table 7.2). Three of the disorders are tissue-invasive, whereas the other three are non–invasive. Specific histopathological criteria are now applied to help differentiate the various forms of fungal rhinosinusitis, which have different clinical characteristics, treatment and prognosis (deShazo *et al.*, 1997; Schubert, 2000). This classification is based on the immunological response of the host to the fungus, and these manifestations may overlap or progress from a non-invasive form into an invasive form if the immunological status of the host changes.

Table 7.2. Forms of fungal rhinosinusitis

Forms of fungal rhinosinusitis	*Macroscopic invasion	**Microscopic invasion	Immuno-compromised host state
• Invasive fungal rhinosinusitis • Acute invasive fungal rhinosinusitis • Chronic invasive fungal rhinosinusitis • Granulomatous invasive fungal rhinosinusitis • Noninvasive fungal rhinosinusitis • Allergic fungal rhinosinusitis eosinophilic fungal rhinosinusitis • Fungal ball • Saprophytic fungal infestation			

*Macroscopic invasion is the apparent infection of paranasal sinuses boundaries diagnosed by clinical examination or by radiological investigations e.g. CT and MRI.

**Microscopic invasion is infection of the mucosa by fungi that can be detected by histopathological examination.

Acute invasive fungal rhinosinusitis

The term acute invasive fungal rhinosinusitis is suggested for immunocompromised patients with invasive fungal rhinosinusitis where the vascular invasion is histopathologically prominent and the time course is less than four weeks.

Chronic invasive fungal rhinosinusitis

Chronic invasive fungal rhinosinusitis usually lasts for more than 4 weeks and vascular invasion is absent or minimal. Patients with chronic invasive fungal rhinosinusitis are only rarely immunocompromised. DeShazo *et al.* (1997) further subdivided chronic invasive fungal rhinosinusitis into cases with granulomatous and nongranulomatous histopathology but there are no apparent differences in prognosis or therapy associated with this distinction.

Granulomatous invasive rhinosinusitis

The distinction of granulomatous invasive rhinosinusitis from chronic invasive rhinosinusitis is somewhat controversial. In the granulomatous form, an enlarging mass is seen in the cheek, orbit, nose, and paranasal sinuses, and proptosis is often a prominent feature. Histopathologically, a granulomatous response is seen with considerable fibrosis. A non-caseating granuloma with a foreign body or Langerhan's type of giant cell may be present, sometimes with

vasculitis, vascular proliferation and perivascular fibrosis. In many cases there are few fungal hyphae present (deShazo *et al.*, 1997; Veress *et al.*, 1973), and the primary agent isolated from these cases is *Aspergillus flavus*. In contrast, the chronic form is characterized by a dense accumulation of hyphae, the presence of vascular invasion, and a sparse inflammatory reaction. *A. fumigatus* can usually be isolated and there is an association with orbital apex syndrome, diabetes mellitus and corticosteroid treatment (Milroy *et al.*, 1989; deShazo*et al.*, 1997). This classification is not recognized by other workers (Washburn *et al.*, 1988; Chakrabarti *et al.*, 1992), and the clinical and pathological distinctions between these two types are not clear-cut. Both forms have a chronic course and predominant orbital involvement. Isolation of different *Aspergillus* species may be the result of geographical distribution and different tissue responses may depend on host immune status. Some workers have further subdivided granulomatous invasive fungal sinusitis into two types; one that has been described from Sudan, and the other as a hypertrophic sinus disease with chronic eosinophilic–lymphocytic granuloma and concomitant allergic fungal rhinosinusitis (AFRS) (Schubert , 2004).

Rowe Jones and Moore-Gillon (1994) described a chronic semi-invasive form of rhinosinisitis that is characterized by sinus expansion and bone erosion but does not show any histological evidence of tissue invasion. Similar examples of semi-invasive pulmonary aspergillosis have also been described and this condition may be a variant of the non invasive type in which the fungal mass destroys the sinus wall by pressure.

Allergic fungal rhinosinusitis

Allergic fungal rhinosinusitis (AFRS) is considered to be a non-tissue invasive fungal process with an allergic/ hypersensitivity response to the presence of extra-mucosal fungi within the sinus cavity. This is possibly analogous to allergic broncho-pulmonary aspergillosis (ABPA). In the diagnosis of AFRS, the detection of fungi in allergic mucin is considered important, although hyphae are sparse in sinus content. This can lead to some confusion in categorising this condition, especially in comparison with two more closely related conditions of eosinophilic fungal rhinosinusitis (EFRS) and eosinophilic mucin rhinosinusitis (EMRS) (Ferguson, 2000; Trasher and Kingdom, 2003). This situation is further complicated by the alternative hypothesis of Ponikau *et al.* (1999), which proposed a different mechanism for AFRS that could be applied universally to encompass chronic rhinosinusitis (CRS) as well. Ponikau *et al.* (1999) used detection of fungi in nasal lavage as a method of diagnosis, and demonstrated the presence of fungi in specimens from 93% of patients with CRS. They did not find type I hypersensitivity to be prevalent in their study group. They suggested that CRS was a cell-mediated response to fungal elements and proposed the new term, EFRS. Therefore there is a question as to whether there is a separate unrecognized form of non-allergic, fungal eosinophilic inflammation that can lead to a similar clinical presentation. Ferguson (2000) claimed that

eosinophilic mucin could be present and cause rhinosinusitis without the presence of fungi. Orlandi *et al.* (2007) recently demonstrated only a few differences between the gene expression profiles from complementary DNA microarray analysis of EMRS and AFS patients. Both of the above conditions are different from normal subjects.

The role of fungi as the target antigen that initiate chronic inflammation in most chronic rhinosinusitis patients is still debated. With the presence of chronic eosinophilic inflammation in chronic rhinosinusitis, an exaggerated reaction to various inhaled antigens is anticipated (Ragab *et al.*, 2006). The role of fungi will be confirmed only when T cells within the sinuses are shown to be actively responding to fungal antigens cultured from the sinus and that their elimination will stop the disease (Ragab and Clement, 2007). The controversy over the definition of AFRS is further complicated by reports of histological invasion in possible cases of AFRS (Schubert and Goetz, 1998; Thakar *et al.*, 2004). Foci of granulomatous inflammation in a AFRS patient with orbital apex involvement have also been reported (Klapper *et al.*, 1997). Further support for hypersensitivity as the cause of AFRS has been suggested by the consistent presence of AFRS with allergic broncho-pulmonary mycosis in the same patients and this has been described as Sino-bronchial Allergic Mycosis (SAM syndrome) (Venarske *et al.*, 2002). These various views and opinions demonstrate the ambiguity in defining AFRS and show that there can be considerable overlap in clinical features, radiological and immunological parameters between AFRS, EFRS and CRS from other causes.

Fungal balls

Fungal balls represent a non-invasive form of fungal rhinosinusitis, and are an accumulation of many fungal hyphae compressed into a mat or ball lying within the sinus lumen extrinsic to the mucosa (deShazo *et al.*, 1997). Only one sinus is usually involved. Fungus balls also are referred to in the literature as mycetomas (deShazo *et al.*, 1997).

Saprophytic fungal infestation

Saprophytic fungal infestation refers to the presence of fungal spores on mucus crusts within the nose and paranasal sinuses. It is defined as the visible growth of fungus within the nasal cavity in an asymptomatic individual and does not refer to fungi that are not visibly growing and can only be detected in culture.

Acute invasive fungal rhinosinusitis: Acute invasive fungal rhinosinusitis is a disorder characterized by acute mycotic infiltration of the mucosa of the nose and paranasal sinuses. The disease is rapidly fatal in 50-80% of patients due to invasion of the orbit and intracranial cavity (Lueg *et al.*, 1996; Kennedy *et al.*, 1997; Gillespie *et al.*, 1998). Overall survival in diabetic patients approaches 80% when the underlying ketoacidosis is corrected (Blitzer *et al.*, 1980). The mortality in patients with bone marrow transplants and cerebral diseases is

considerably worse (Blitzer *et al.*, 1980). Orbital and intracranial involvements carry a poor prognosis. The disease occurs primarily in immunocompromised patients in whom the neutrophilic response is absent or impaired. Suryanarayan Rao *et al.* (2006) and Chopra *et al.* (2006) each reported the disease in 5 immunocomptent patients. All the reported cases in immunocomompetent subjects have so far been in India. It is most commonly associated with *Mucor* species but it has also been seen in association with *Aspergillus* species. Fungal sinusitis secondary to *Pseudallescheria boydii* infection is rare, and only 26 cases have been reported in the literature. *Mucor* species have a predilection to internal lamina of the arterial blood vessels and later involve lymphatics and veins (Bates and Mims, 2006).

Mucormycosis is used to describe the condition when it is due to one of the four families of the order Mucorales. Rhinocerebral mucormycosis is the used to describe mucormycosis where there is an involvement of the brain in addition to the paranasal sinuses (Gillespie *et al.*, 1998).

Clinical presentation

The initial critical step in diagnosis is the proper identification of people at risk (Table 7.3). Significant secondary risk factors have been identified. Previously some 70% of cases have been associated with patients with diabetes mellitus. Hyperglycemia and ketoacidosis enhance tissue invasion and fungal growth due to ketoacidosis leading to a decrease in phagocyte activity against polymphonuclear leukocytes (Iwen *et al.*, 1997).

Table 7.3. Risk factors for invasive fungal sinusitis

-Low granulocytic count (below 500cells/ml)	-Diabetes mellitus (poorly controlled type 1)
-Acute leukemia	-Immunologic disorders (CD4<50/mm^3)
-Lymphoma	-Systemic steroids
-Multiple myloma	-Renal failure
-Cytotoxic drugs	-Malnutrition
-Irradiation	-Gastroenteritis
-Transplantations	-Burn

The initial symptom in up to 90% of patients is a fever of unknown origin that has not responded to 48 hours of appropriate broad spectrum intravenous antibiotics, although the absence of fever does not rule out the disease (Bent and Kuhn, 1994). Localised symptoms of CRS are variably present in 20-60% of patients (Iwen *et al.*, 1997; Gillespie *et al.*, 1998). Most patients, however, have both fever and at least one local symptom (Gillespie *et al.*, 1998). Late signs and symptoms include loss of visual acuity, ophthalmoplegia, proptosis, and change in mental status, focal neurological signs, and seizures.

Physical examination

A thorough nasal endoscopy should be performed in any at risk patients. The disease is thought to originate in the nasal cavity in most cases before extending into the paranasal sinuses or the surrounding structures such as the orbit palate, septum (Fig. 7.1) and intracranial structures or to the skin of the nose. If the disease is diagnosed while it is confined to the nasal cavity, a complete endoscopic resection can be expected to have a positive impact on patient survival (Gillespie *et al.*, 1998). The most consistent clinical finding is an alternation of the nasal mucosa. Discoloration, granulation and ulceration often replace the normal pale pink mucosa (Gillespie *et al.*, 1998). Mucosal discolorations are variable and may be grey, green, white, or black (Fig. 7.2). White colours indicate tissue ischemia secondary to angiocentric invasion, while black colourations are a late presentation of tissue necrosis. Mucosal abnormalities were most commonly seen on the middle turbinate (67% of patients) followed by the septum (24%), palate (19%) and inferior turbinate (10%) (Gillespie *et al.*, 1998). Decreased mucosal bleeding or sensation should also be noted using a 27-gauge needle inserted using nasal endoscopes as these may be a sign of fungal invasion.

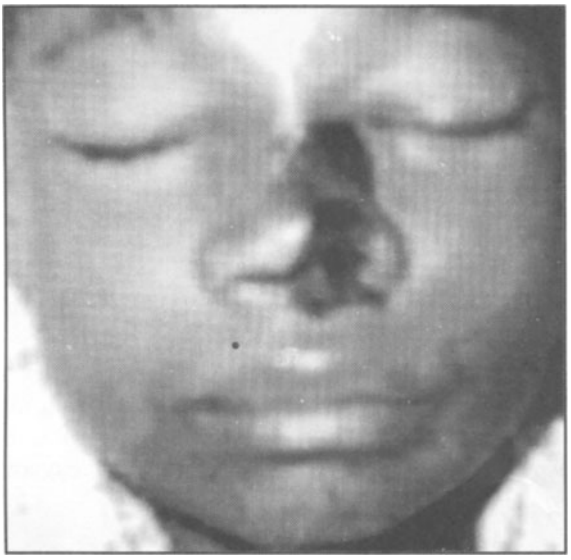


Fig. 7.1. Crusty dark black necrotic skin of the left dorsum, ala, tip and columella of the nose in neglected acute invasive fungal rhinosinusitis in a child with acute leukemia.

A thorough head and neck examination should be performed on all patients in addition to the endoscopic nasal evaluation. The patients' level of consciousness and awareness can give a rough evaluation of central nervous system function. A full cranial nerve examination should be undertaken with particular attention to facial sensation, extra-ocular muscle function, afferent pupillary reflex and visual acuity.

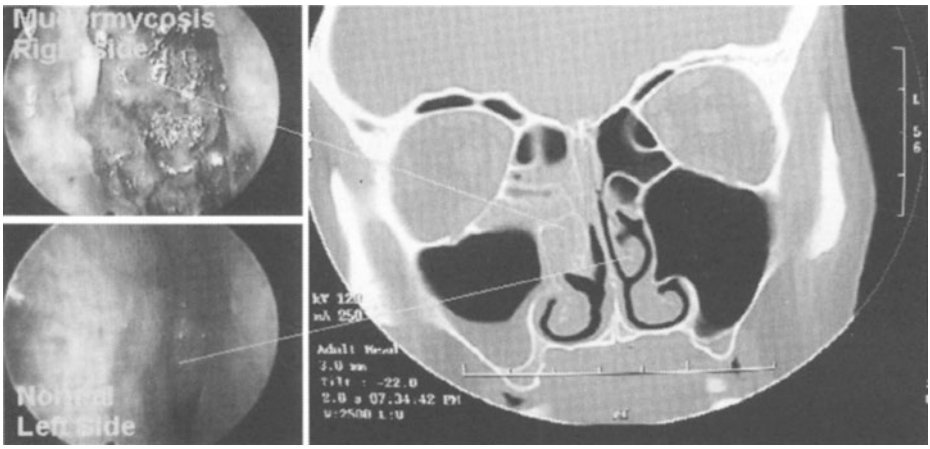


Fig. 7.2. Acute invasive fungal rhinosinusitis in male patient with uncontrolled type 1 diabetes mellitus; endoscopic view shows middle meatus granulation tissue with gray, white and black mucosal discolorations that indicate tissue ischemia while the left side is normal and CT revealed right soft tissue density of the right anterior ethmoid sinuses, middle meatus and middle turbinate

Imaging

Fine cut (2mm) CT scans of the nose and paranasal sinuses should be obtained in both axial and coronal cuts as soon as the disease is considered. Intravenous contrast is helpful in delineating periorbital or dural inflammation. Early mucosal thickening of the pranasal sinuses should be noted (Fig. 7.2). Nodular thickening of the sinus mucosa and spotty destruction of the bony walls may be seen. Orbital involvement through the lamina papyracea is a late manifestation. Inflammatory involvements of the ophthalmic vein and artery have been described as specific indications of orbital apex involvement in mucormycosis (Marple, 1999). Normal CT is not uncommon in invasive fungal disease (12%) (Gillespie *et al.* 1998), and emergent MRI is required in such patients and is especially helpful in evaluating the orbit, cavernous sinuses and brain (Gamba *et al.*, 1986).

Histopathological diagnosis

Biopsy at the bed side using nasal endoscopy may yield useful information after excluding bleeding diathesis (platelet count > $60x10^9$). Biopsy of the suspected site allows accurate diagnosis. Middle turbinate biopsy has overall sensitivity of 86% and specificity of 100% (Miller *et al*, 1982). Limited size biopsy by 2mm cup forceps followed by silver nitrate cautery and insertion of absorbable gelatin sponge or cellulose packing may be used to decrease the risk of bleeding.

The histopathological criteria (Table 7.4) can be identified after frozen section or permanent section with Gomori Methnamine silver stain. Frozen-section biopsy is a useful tool for rapid and effective diagnosis in patients with suspected AIFRS (Ghadiali *et al.*, 2007). A portion of the specimen should be sent for culture to obtain epidemiological information and to determine the appropriate antifungal medication.

Table 7.4. Histopathological criteria for invasive fungal rhinosinusitis

- Hyphae forms within the submucosa with or without angiocentric invasion
- Tissue necrosis with minimal host inflammatory cell infiltration.

When *Mucor* species are involved histopathology will show irregularly shaped non septate hyphae of 6-50 μm that branch at right angles. Aspergillus hyphae are 2.5-5 μm, septate and typically show 45° dichotomous branching.

Treatment

The standard treatment of acute invasive fungal rhinosinusitis is by a combination of antifungal antibiotics and aggressive surgical debridment (Drakos *et al.*, 1993; Choi *et al.*, 1995). More specific treatment regimes are detailed in Table 7.5.

Table 7.5. Specific treatment for acute invasive fungal rhinosinusitis:

Correction of any underlying predisposing and immunological disorders
Intravenous antifungal therapy
Wide surgical debridement of all tissues involved
Establishment of adequate sinus and orbital drainage
Continuous monitoring to avoid recurrence and persistence of the disease

The antifungal Amphotricin B (>1.25mg/kg per day) is the drug of choice for treatment of such disease. One report found an initial 85% response rate to amphotricin B, however, 615 of the respondents subsequently died from invasive aspergillosis (Goering *et al.*, 1988). Amphotricin B can have acute side effects including chills, fever, headache, thrombocytopenia and nausea and vomiting. Long term reactions includes nephrotoxicity, ototoxicity, hypokalaemia, and bone marrow depression. A standard regime should start with a 1 mg test dose on the first day of therapy followed by progressive escalation of the dose by daily increments of 5mg until the required daily dose is reached. Patients intolerant for amphotrine B may be candidates for liposomal amphotricin B which is used at 5mg/kg/day (Leenders *et al.*, 1998). The major factor limiting the use of the liposomal form is its higher cost. The new triazole antifungal agent voriconazole has a broad spectrum of activity against fungal pathogens including *Aspergillus* species. This has been investigated by Baumann *et al.* (2007) a study in four patients with clinical, radiological and histological signs of invasive sphenoidal aspergillosis.

The surgical approach has changed over the years due to a better understanding of the disease. Extensive surgical resection in the form of radical maxillectomy, craniofacial resection, and orbital exentration have given way to limited endoscopic sinus debridement. Radical resection rarely achieved negative margins or improved long term survival (Kennedy *et al.*, 1997; Gillespie *et al.*, 1998). Surgery should be performed as soon as possible, and postoperative irrigation of the sinus with amphotricin B can give better control and prognosis (Vener *et al.*, 2007). Recently some clinicians have used retrobulbar amphotericin B injections for treatment of invasive sino-orbital aspergillosis. Post operative debridement and follow up is essential and occasional biopsy may be required (Wakabayashi *et al.*, 2007).

Other additional important measures such as granulocyte colony stimulating factor (GCSF) have been shown to be efficacious for promoting bone marrow recovery in neutropenic patients. Couch *et al.* (1988) reported on two patients who improved following hyperbaric oxygen therapy. Other measures include white blood cell transfusion, and bone marrow transplantation.

Significant complications of IFRS can occur after medical remission and recovery of immune competence. Patients with IFRS should be followed long term until remucosalization of the sinuses, resolution of crusting, and cessation of bony sequestration has occurred (Otto and Delgaudio, 2006).

Prevention

It is suggested that all patients who plan to undergo bone marrow transplantation are screened for symptoms and signs of rhinosinusitis. If symptoms are present CT and endoscopic assessment is an essential request. Kavanagh *et al.* (1991) reported that 42% of patients with leukemia had abnormal radiographs and eight of 36 cases operated on were found to have fungal sinusitis. Medical treatment should be started and CT and nasal endoscopy should be repeated.

Chronic invasive fungal rhinosinusitis

Chronic invasive fungal rhinosinusitis is difficult to categorise discretely, the clinical course may span months or years, and it is described as being more common in immunocompetent patients (Washburn *et al.*, 1988). A chronic granulomatous form has been reported in Sudan, and the non granulomatous forms occur mainly in diabetic patients with type 2 diabetes.

Clinical presentation

Patients are generally healthy but many have a history of CRS symptoms, upper respiratory allergies or nasal polyposis. Symptoms may take months or years to appear. Proptosis is the most common presentation of the granulomatous form in Sudan (Veress *et al.*, 1973). Invasion of the maxillary floor may produce

palatal erosions. Erosion of the cribriform plate may cause chronic headache, seizures, decreased mental status, or focal neurologic findings. Extension through the sphenopid sinus may lead to orbital apex syndrome (Washburn *et al.*, 1988), and extension to the ptyrigopalatine fossa may cause cranial nerve defects.

On intranasal examination severe nasal congestion and polypoid mucosa may be noted. There may be soft tissue mass that can be either mucosally covered or ulcerated with overlying debris or dried secretions (Milosev *et al.*, 1969). A faint yellow hue may also be present.

Imaging

CT is recommended and will show significant soft tissue thickening and evidence of altered adjacent bone. It is important to note that both chronic invasive fungal rhinosinusitis and AFRS can cause bony erosion or expansion suggesting potentially invasive process (Fig. 7.3). Silverman and Mancuso (1998) noted that soft tissue infiltration of peri-antral fat planes around the maxillary sinus provides early evidence of invasive fungal rhinosinusitis. MRI is useful for assessment of dural involvement.

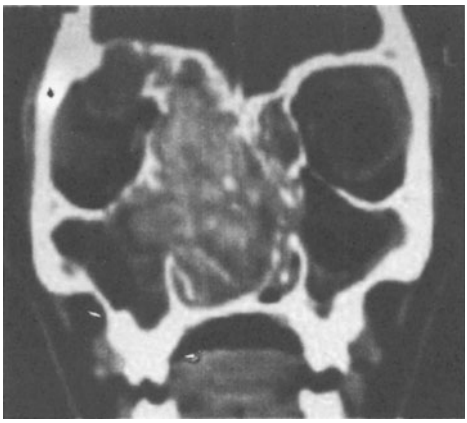


Fig. 3. Coronal CT showing heterogeneous soft tissue density with bony erosion and expansion suggesting potentially invasive process of chronic invasive fungal rhinosinusitis

Pathogens

Aspergillus species are the most common organisms involved and *A. flavus* is the most common cause in Sudan (Milosev *et al.*, 1969). The dry climate, a high airborne spore count, high atmospheric dust and frequent sandstorms were the main precipitating factors in Sudan. Experimental invasive infections in mice show *A. flavus* to be 100-fold more virulent than *A. fumigatus* in terms of the amount of inoculum required. In addition, *A. flavus* produces aflatoxins, the most toxic and potent hepatocarcinogenic natural compounds known. Accurate species identification within *Aspergillus flavus* complex is difficult due to overlapping morphological and biochemical characteristics (Hedayati *et al.*, 2007). Chronic

invasive fungal rhinosinusitis has also been associated with species of *Mucor, Alternaria, Curvularia, Bipolaris, Candida, Dreschslera, Sporothrix schenckii* and *Pseudallescheria boydii* (Washburn *et al.*, 1988; Schwartz *et al.*, 1997; Hedayati *et al.*, 2007).

Pathology

Diagnosis is first confirmed by histopathology on either frozen or permanent section. Veress *et al.* (1973) described the gross appearance as firm, hard, rubbery, fibrous, grayish white mass with an irregular surface (Washburn *et al.*, 1988). They described three variants: proliferative (granulomatous pseudo tubercles in a fibrous tissue stroma), exudative-necrotizing (with prominent foci of necrosis) and a mixed form. Dechazo *et al.* (1997) typified granulomatous as granulomatous composed of eosinophilic material surrounded by fungus, giant cells and palisading nuclei and non granulomatous characterized by tissue necrosis with little inflammatory infiltrates and dense hyphal accumulation like fungus balls.

Treatment

This spectrum of diseases is best handled through a combination of medical and surgical treatment. The surgery may be approached through endoscopically minimally invasive techniques or traditional open surgery. Owing in part to their vascularity the periorbita and dura appear to be barriers to the spread of the disease while bone is not. The choice of the antimycotic agent is based on the type of fungus. Amphotericin is the agent typically initiated. Recurrence is not uncommon despite antifungal therapy following surgery (Weber *et al.*, 1987). Gumaa *et al.* (1992) used 100mg itraconazole twice daily for 6 weeks with good results. If clinical, radiological and serological cure was achieved the dose reduced to 100mg daily for 12-19 months. Follow ups with CT every month and endoscopy every 2-3 months are recommended.

Fungal balls of the paranasal sinuses

Fungal balls at present are the most frequent cause of chronic unilateral sinusitis that is unresponsive to normal medical management. It is also the most frequent form of fungal sinusitis, especially in Europe (Morpeth *et al.*, 1996). It can be defined as the presence of a mycelial mass that remains confined to the lumen of the sinus cavity, usually in the maxillary antrum, which appears as chronic sinusitis or recurrent sinusitis that doesn't react to antibiotics in apparently immunocompetent individuals. In the literature the terms aspergilloma and aspergillosis are also used for paranasal fungal sinus balls (Dhong *et al.*, 2000).

Aetiology

Fungal sinus balls are usually caused by fungi that are abundant in the air and biologically apt to implantation, and these include species of *Aspergillus,*

Mucorales, Fusarium and some dematiacious hyphomycetes. The most common fungi vary according to country and geographic region, and while *Aspergillus fumigatus* is the most frequent agent in Europe (Finby and Begg, 1975), *Aspergillus flavus* is more frequent in India and Sudan. *Aspergilli are* ubiquitous fungi, which are cosmopolitan. In man, they are saprophytes in the nasal fossae, the mouth and the digestive tract (Eloy *et al.*, 1997).

There are two modes for fungi to reach the involved sinuses which either odontogenic or through the airway. The presence of pathological secretions in viral or bacterial super-infection in combination with genetically determined growth rhythm of the fungus produces a periodic growth of peripherally oriented fungal hyphae. This gives the fungal mass the characteristic onion like appearance on histology sections (Stammberger, 1985). The restriction of fungal balls to certain sinuses can be explained by the lack of viability and sporulation of the fungi *in situ*, together with possible sinus ostium occlusion (Ragab *et al.*, 2006).

Epidemiology

Older individuals appear to be more susceptible, and in a Mayo clinic review the incidence among surgical specimens coded as inflammatory sinusitis was 3.7% for Fungal ball, compared to 6.9% for allergic fungal rhinosinusitis and 0.003% for invasive fungal sinusitis (Ferreiro *et al.*, 1997).The average age of the cases reported was 64 years, in a range of 28-86 (Ferreiro *et al.* 1997; deShazo *et al.* 1997; Klossek *et al.* 1997). There is also consistently a female predominance of about 64% (Ferguson , 2000 a).

Diagnosis

Primary diagnosis is by direct histopathological and mycological examination of the sinus content and its mucosa complemented by culture to determine the fungal species. Failure of the fungus (from the fungal ball itself) to grow on fungal culture is common, with fungal culture rates of 23% in one series (deShazo *et al.*, 1997) and 50% in other series (Klossek *et al.*, 1997). The fungi reported to cause fungal balls include *Aspergillus fumigatus, Aspergillus flavus, Alternaria sp., Rhizopus microsporus and Pseudallescheria boydii* (Ragab *et al.*, 2006). Also, because of the absence of chronic inflammation that can upload the fungal existence to allergic fungal sinusitis (AFS), most cases of fungal balls did not change to AFS or eosinophilic fungal rhinosinusitis with such prolonged exposure to such fungi (Ragab *et al.*, 2007).

Clinical presentation

Fungal balls are usually confined to one sinus, and the maxillary sinus is the most common site of infection (87.8%), followed by the sphenoid (Dufour *et al.*, 2006), the ethmoid and the frontal sinus (Klossek *et al.*, 1997). Rarely multi-sinuses involvements can occur (Fig. 7.4). The clinical presentation is that of asymptomatic long standing chronic sinusitis, often non-painful, associated with

a unilateral greasy sticky purulent rhinorrhea unresponsive to appropriate medical treatment (deShazo *et al.*, 1997). Eventually, the patients can complain of cacosmia. Sometimes more non-specific complaints are also noted such as unilateral posterior rhinorrhea, alternating nasal obstruction or a night time cough in decubitus (Eloy *et al.*, 1997). The sensitivity of the unilateral oily gelatinous discharge was determined by Dhong *et al.*. (2000) as 23% with predictive value of 100%.

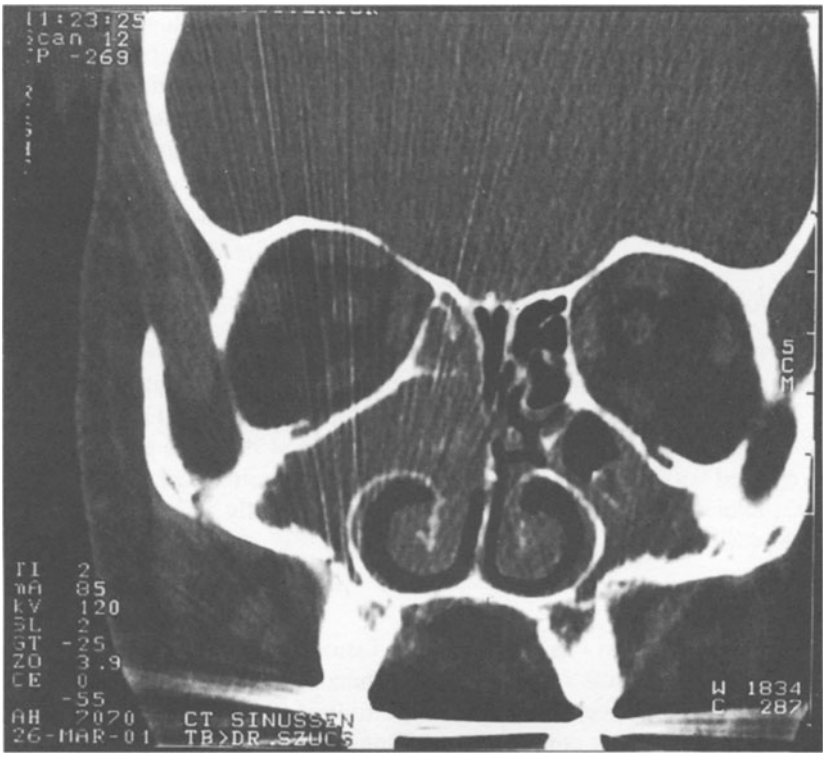


Fig. 7.4. CT scan coronal image of the paranasal sinuses showing complete opacification of the right maxillary and ethmoid sinuses with spontaneous hyperdense zone. Also there exist partial opacification of the left maxillary sinus with also central hyperdense zone (bilateral fungal balls).

Diagnostic development

Nasal and sinus endoscopies may reveal purulent secretions, blackish crusts, even polyps of the middle or superior meatus that may proceed or promote fungal balls and lead to hypoxic anaerobic media (Eloy *et al.*, 1997). In 1/3 of cases, endoscopy is normal (Ferguson, 2000 a).

CT scanning of the sinuses is very evocative in the majority of the cases and more sensitive than routine sinus films (Klossek *et al.*, 1997). Typically, the disease is unilateral and in 94% of the cases in a study by Klossek *et al.* (1997)

the fungal ball only involved one sinus. The fungal ball appears most often as a heterogeneous opacity in the interior of a sinus, without evidence of any destruction and with spontaneously hyper-dense areas in 25-50% of cases (Fig. 7.4) (Klossek *et al.*, 1997). With fungal balls the sensitivity of the CT is 62% and 99% specificity, although there can be 22% false positive and 2% false negative results (Dhong *et al.*, 2000). Stammberger (1985) has suggested that deposits of calcium phosphate and calcium sulphate form in the region of the mycetoma. In certain cases zones of bony deformation in the form of thinning or displacement can be seen in CT scans (deShazo *et al.*, 1997) which is mainly due to mass effect but never to osseous invasion by the fungus.

With MRI, one sees attenuation of the signal in weighted mode T2, which appears to be secondary to the presence of elevated levels of magnesium, manganese and iron in the fungal concretions (Fig. 7.5).

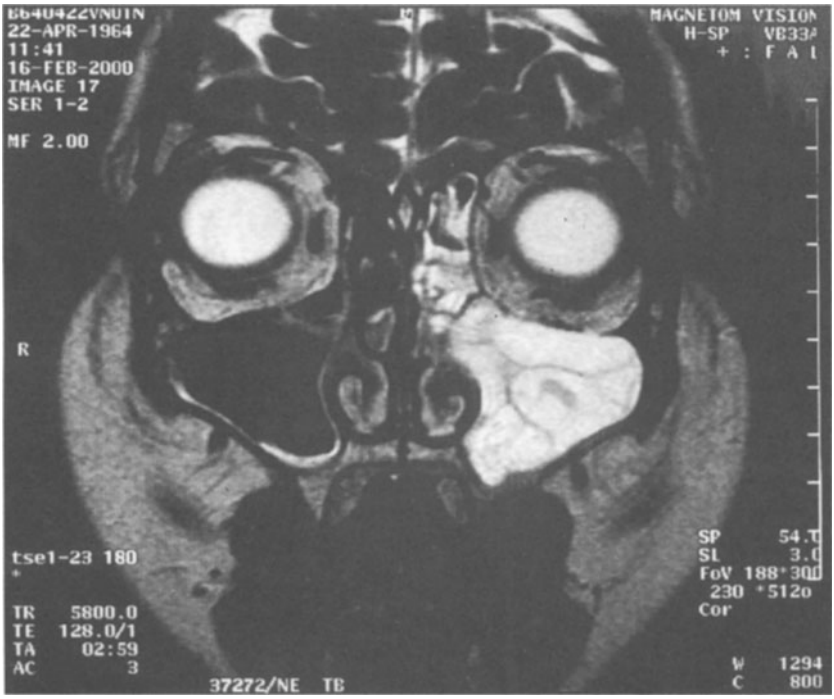


Fig. 7.5. T2-weighted MRI coronal section of the paranasal sinuses showing area of hypointensity in the left maxillary sinus

Histopathology

DeShaazo *et al.* (1997) suggested that diagnostic criteria for mycetoma (Fungal ball) included no histological signs of fungal invasion of sinus mucosa, blood vessels or bone, and that dense and compact aggregations of septate hyphae were present lying in adjacent sinus mucosa but not infiltrating it.

Table 7.6. Criteria for diagnosis of fungal balls:

1-Presence of partial or total opacification of one or more sinuses with or without micro-calcification on routine sinus CT.

2-**Macroscopic symptoms**

Adherent and friable clay like material of greenish, brownish, or greyish colour sometimes appearing as a thick with putty like material (Ferreiro *et al.*, 1997).

3-**Hisopathological analyses**

With haematoxylin and eosin (H&E) staining: Varying degrees of inflammatory infiltrate in the sinus mucosa adjacent to fungal element (Fig. 7.6) (Fig. 7.7) can be identified composed of lymphocytes, plasmocytes, mastocytes, neutrophils and eosinophils. Also there is no inflammatory granuloma centered in the hyphae or allergic mucin.

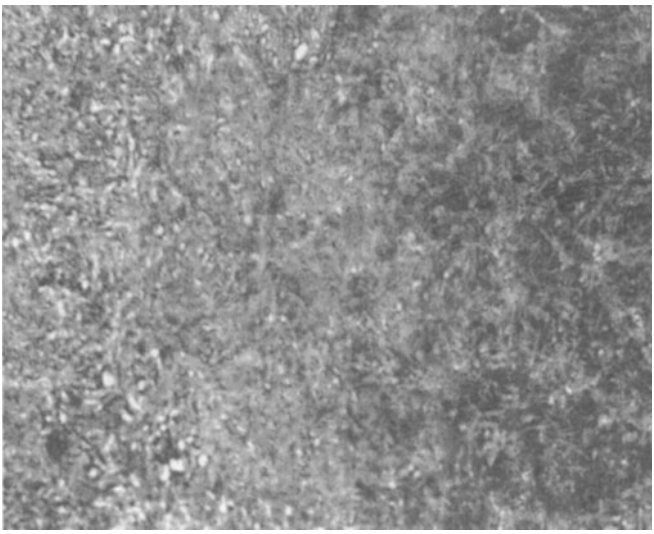


Fig. 7.6. Histopathology showing a tangled mat of hyphae characteristic of a fungal ball (H&E staining)(Original magnification x100). By culture *Aspergillus sp* and *Rhizopus microsporus* were identified

With PAS and GMS staining: No histological signs of fungal invasion of sinus mucosa, blood vessels or bone. Also dense and compact aggregation of septate hyphae lying in adjacent sinus mucosa but not infiltrating (Fig. 7.8). The morphology of the filaments can help in the diagnosis of the species. *Aspergillus* species are characterized by densely tangled hyaline hyphae of 2-5 μm diameter with classically 45°dichotomous branching suggestive of mistletoe.

Mycological examination

Mycological examination can identify the causative species, and consists of direct examination and culture of sinus contents. This procedure is less sensitive than histopathology and only detects 30-50% of positive cases with high levels of false positive and negative results (Ragab *et al.*, 2006).

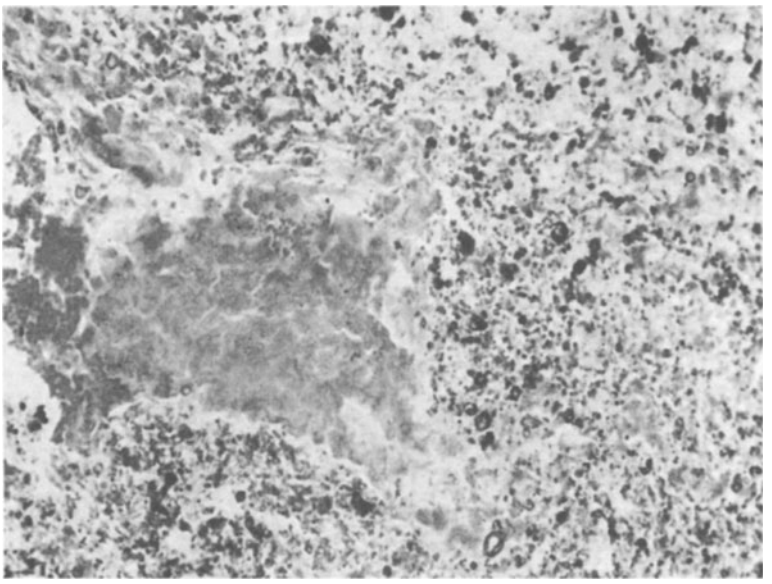


Fig. 7.7. Histopathology of fungal ball showing mixed Aspergillus sp (black arrow) and dematiaceous species (*Alternaria alternata*) which are brown in colour. They appear brown because their cell wall contain melanin and possess a brown to black pigment (Original magnification x 100).

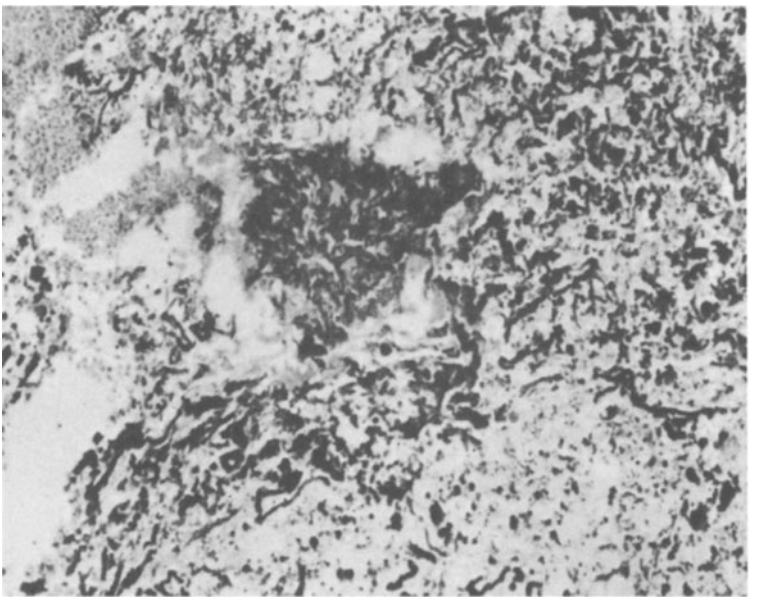


Fig. 7.8. Histopathology of the same fungal ball specimen stained with GMS staining showing the dark colored hyphae of both species *Aspergillus* and *Alternaria* (Original magnification x100)

Among the various *Aspergillus* species that cause fungal balls in human (*A. fumigatus*, *A. flavus* and *A. niger*) (Fig. 7.9) *A. niger* is the most common (Dufour *et al.*, 2006).

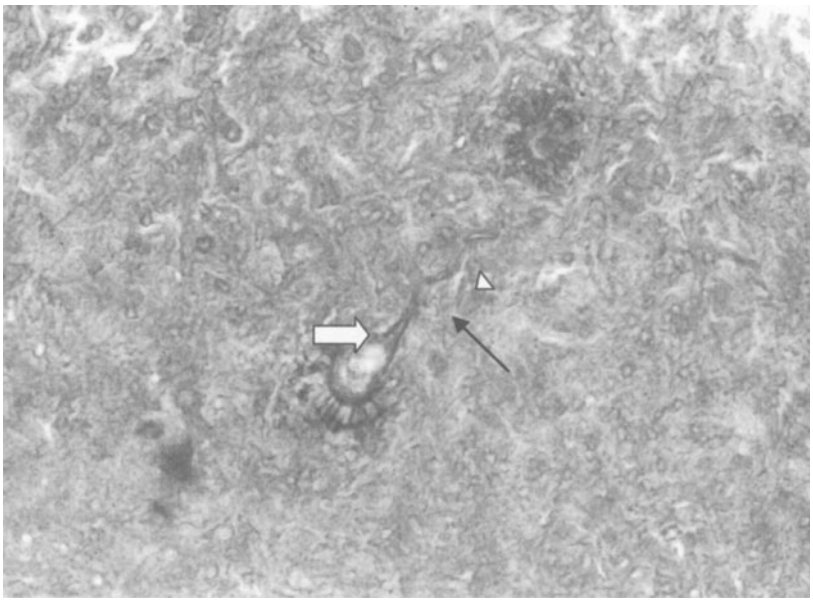


Fig. 7.9. Histopathology section of fungal ball of the maxillary sinus showing spore formation within maxillary sinus by *Aspergillus niger*. The vesicle can be shown (black arrow) and also the spore forming cells (white arrow) and the supporting stalk conidiophore (white arrow head) (H&E stain, original magnification x 300)

The diagnosis is often made through clinical findings with the collaboration of medical imaging. In cases of doubt, sinus endoscopy permits the observation of blackish concretion overlying the mucosa and can provide a specimen for histopathological and fungal examination.

Treatment

An intra-operative dirty clay like mass is a specific indicator of a fungal ball. The sensitivity, predictive value and positive rate of this finding are 10%, 99% and 83% respectively (Dhong *et al.*, 2000).

Treatment is usually surgical and must be radical, consisting of the extirpation of the fungal mass and re-establishment of optimal drainage and ventilation conditions in the affected sinuses. At the present time, this surgery is performed through an endonasal approach under endoscopic control (Klossek *et al.*, 1997). In cases where it is not possible to completely extirpate the mycelial mass endonasally; an external approach may be required, although in recent years this has rarely been undertaken.

Allergic Fungal Rhinosinusitis/eosinophilic fungal rhinosinusitis

First reported by Miller *et al.* (1981), AFRS is a noninvasive fungal rhinosinusitis with associated hyperplastic eosinophilic sinusitis that represents an allergic and immunological response to the presence of fungal hyphae found within the sinus cavities. A wide range of clinical, radiographic and immunological features have been used to define the condition, and have led to the development of a number of diagnostic criteria (Loury *et al.*, 1993; Bent and Kuhn, 1994). Patients with AFS uniformly show five characteristics: gross production of eosinophilic mucin-containing noninvasive fungal hyphae, nasal polyposis, characteristic radiographic findings, immunocompetence and allergy to fungi (Bent and Kuhn, 1994). Several diagnostic criteria for AFS are primarily histopathological and obtained from sinus surgery specimens (Table 7.7) (Schubert and Goetz, 1998).

Table 7.7. Histopathological diagnostic criteria for allergic fungal sinusitis (all criteria must be met) [adapted from (Schubert and Goetz, 1998)]

1. Characteristic allergic mucin is seen histopathologically and/or grossly
2. Fungal stain is positive for hyphae within the allergic mucin, but not in the mucosa, or the surgical sinus fungal culture is positive in an otherwise characteristic patient
3. The sinus mucosa demonstrates eosinophilic–lymphocytic inflammation without evidence for tissue necrosis, granulomas or fungal invasion
4. Other fungal diseases are excluded

In 1999, a hypothesis of CRS was proposed by Ponikau and his co-workers. that suggested colonizing fungi in sinus mucus played a much broader role in the pathogenesis of CRS. In a sensitive culture technique they found that 93% of 101 patients with CRS gave positive fungal cultures from nasal lavage. A new regime for all CRS patients limiting the diagnostic criteria to the presence of fungi and the presence of allergic mucus-containing clusters of eosinophils was suggested.

Classic AFRS

Over the course of the past 25 years, AFRS has emerged as a clinically distinct subset of CRS. AFRS possesses characteristic clinical, radiographical, pathological, and immunological features.

Unlike invasive forms of fungal rhinosinusitis, AFRS is characterised by the potential for colonizing fungi to elicit allergic mucosal inflammation in the absence of invasion. The ability of fungi or, more specifically, protein components of fungi to elicit IgE-mediated allergic mucosal inflammation is well documented (Horst *et al.*, 1990). Moreover, when those sensitized individuals are placed in environments of high fungal exposure, symptoms of airway hyperresponsiveness increase significantly over those of nonsensitized individuals in similar situations (Downs *et al.*, 2001). Virtually all studies of the

pathophysiology of AFRS have been based on the premise that IgE mediated allergy to one or more fungi underlie the disease, with the predominant finding of eosinophil-predominant tissue infiltration akin to late-phase allergic inflammation. In this way AFRS has features quite similar to those of allergic bronchopulmonary aspergillosis (ABPA) (Manning *et al.*, 1989).

Table 7.8. Primary features of ABPA and AFRS [adapted from (Manning *et al.*, 1989)]

Feature	ABPA	AFRS
Allergic mucin with non invasive fungal hyphae	Yes	Yes
Atopy	Yes	Yes
Elevated total IgE	Yes	Yes
Allergy skin test are positive for fungal organism	Yes	Yes
Elevated fungal specific IgG	Yes	Yes
Elevated fungal specific IgE	Yes	Yes
Serum precipitins	Yes	No
Peripheral eosinophilia	Yes	No
Change in total IgE prognostic	Yes	Yes

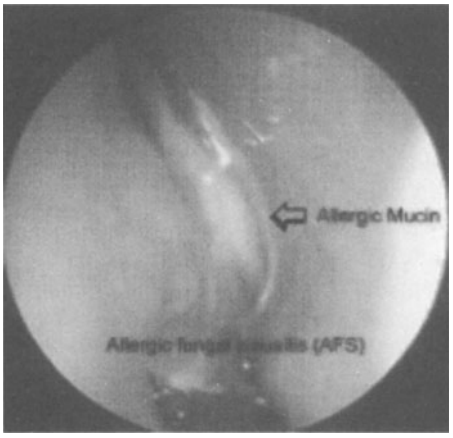


Fig. 7.10. Endoscopic view in a patient with allergic fungal rhinosinusitis showing allergic mucin with nasal polyposis

History and Physical Findings

Occasionally, the presentation of AFRS might be dramatic, giving rise to acute visual loss, gross facial dysmorphia (described below), or complete nasal obstruction (Manning *et al.*, 1989) but more often, the presentation of AFRS is subtle. Patients typically complain of gradual nasal airway obstruction and production of semisolid nasal crusts that match the gross description of allergic fungal mucin. The development of nasal airway obstruction might have been so

gradual that the patient is unaware of its presence. Pain is uncommon among patients with AFRS and suggests the concomitant presence of a bacterial rhinosinusitis (Marple, 1999; Manning and Holman 1989). In contrast to the often subtle symptoms of AFRS, physical findings are often more remarkable. The range of physical findings on examination is typically broad, ranging from nasal airway obstruction resulting from intranasal inflammation, allergic fungal mucin and polyposis (Fig. 7.10) to gross facial disfigurement and orbital or ocular abnormalities (Marple *et al.*, 1999). Progressive facial deformities such as proptosis, telecanthus and malar deformity can occur, especially in paediatric patients. The incidence is about 20% in some series (Bent and Kuhn, 1994). At present the most helpful clinical tools are nasal endoscopy and a mucosal staging system (Table 7.9) (Kupferberg *et al.*, 1997; Kuhn and Javer, 2000):

Table 7.9. Nasal endoscopy mucosal staging system

Stage 0:	No mucosal oedema or allergic mucin.
Stage 1:	Mucosal oedema with or without allergic mucin.
Stage 2:	Polypoid oedema with or without allergic mucin.
Stage 3:	Sinus polyps with fungal debris /mucin.

Radiological Findings

The slow accumulation of allergic fungal mucin provides unique and rather predictable characteristics to the disease. AFRS, although bilateral in 51% of the cases reviewed, caused asymmetric involvement of the paranasal sinuses in 78% of the cases (Fig. 7.11, 7.12). Bone erosion and extension of disease into adjacent anatomical areas was encountered in 20% of the patients and was more likely to occur in the presence of bilateral advanced disease (Fig 7.12).

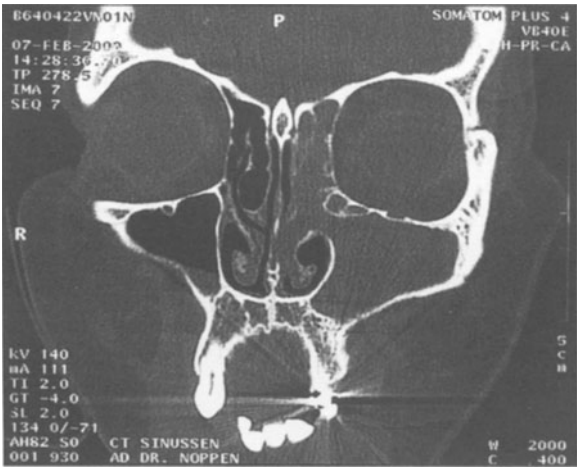


Fig. 7.11. Coronal CT image of paranasal sinuses in AFRS patient reveals unilateral left maxillary and ethmoid sinuses opacification

Expansion, remodelling, or thinning of involved sinus walls was common (and was thought to be due to the expansile nature of the accumulating mucin). These finding were corroborated by Nussen-baum *et al.* (2001) who found demineralization of bone in approximately 20% of the subjects. Heterogeneous areas of signal intensity within paranasal sinuses filled with allergic fungal mucin are frequently identified on CT scans (Fig. 7.12). Although these findings are not specific for AFRS, they remain relatively characteristic of the disease and might provide preoperative information supportive of a diagnosis of AFRS (Mukherji *et al.*, 1998).

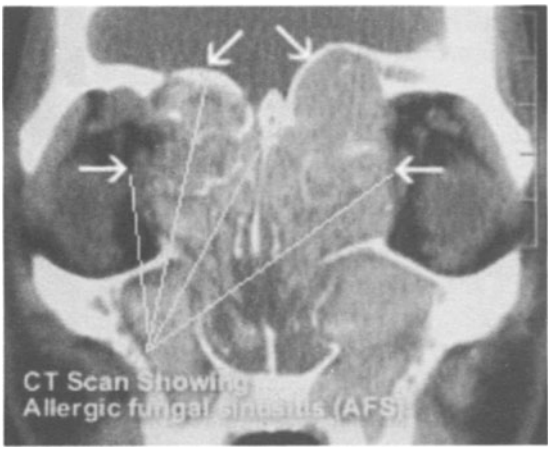


Fig. 7.12. Coronal CT image of paranasal sinuses in AFRS patient showing bilateral multiple sinuses heterogeneous density with bone erosion and macroscopic extension of disease into adjacent anatomic areas (orbit and anterior cranial fossa). This finding was encountered in 20% of the patients and was more likely to occur in the presence of bilateral advanced disease

Current evidence points to the presence of accumulations of metals (e.g. iron and manganese) and calcium salt precipitation within allergic fungal mucin as the most likely cause of these radiographic findings (Mukherji *et al.*, 1998; Zinreich *et al.*, 1988).

MRI can also provide information useful in the preoperative identification of allergic fungal mucin. This effect is more pronounced on T2-weighted images as a result of prolonged magnetic field relaxation times. The high protein and low water concentration of allergic fungal mucin, coupled with the high water content within surrounding edematous paranasal sinus mucosa, gives rise to rather specific magnetic resonance characteristics. Combined CT and MRI findings provide a radiographic appearance that is highly suggestive of AFRS (Mabry and Manning, 1995; Manning *et al.*, 1997).

Immunological testing

A study by Manning and Holman (1998) compared eight patients with culture positive *Bipolaris* species AFRS with ten control subjects with CRS. Both

groups were evaluated by (1) RAST and ELISA inhibition to *Bipolaris* species–specific IgE and IgG antibodies and (2) skin testing with *Bipolaris* species antigen. All eight patients with AFRS gave positive skin test reactions to the *Bipolaris* species antigen, as well as positive RAST and ELISA inhibition results to *Bipolaris* species– specific IgE and IgG. In comparison, eight of the ten control subjects gave negative results on both skin and serologic testing.

Several other studies have also demonstrated a positive correlation between skin test and *in vitro* (RAST) responses for both to fungal and non-fungal antigens in patients with AFRS (Mabry and Manning, 1995; Manning and Holman, 1998). Moreover, patients with AFRS appear to demonstrate a broad sensitivity to a number of fungal and non-fungal antigens (Manning *et al.*, 1993).

Sensitivity to numerous fungi has been shown by both *in vitro* (RAST) and *in vivo* (skin testing) methods; although generally only a single fungus is isolated from culture of the corresponding allergic fungal mucin. Chrzanowski *et al.*, (1997) identified the presence of an 18-kd protein in allergic mucin obtained from patients with AFRS, which might represent a fungal pan antigen.

Total IgE values are also generally increased in patients with AFRS, often to more than 1000 IU/mL, and have been proposed as a clinically useful indicator of AFRS disease activity (Manning *et al.*, 1993). In some cases fungus-specific IgG precipitins have also been detected analogous to those described in allergic bronchopulmonary aspergillosis.

Histological Characteristics of Allergic Mucin

Production of allergic mucin is considered diagnostic for AFRS. Grossly, allergic mucin is thick, tenacious, highly viscous and its colour can vary from light tan to brown or dark green (*et al.*Corey, 1992; Chrzanowski *et al.*, 1997). It is the mucin, rather than paranasal sinus mucosa, that provides the histological information necessary to diagnosis AFRS (Torres *et al.*, 1996; Schnadig *et al.*, 1999). Examination of mucosa and polyps obtained from the paranasal sinuses involved show signs of chronic inflammation, usually with an abundance of eosinophils (Fig. 7.13). Pathological examination of these tissues is required to establish that fungal invasion is not present (Torres *et al.*, 1996).

Histological examination of allergic mucin will show characteristic branching non-invasive fungal hyphae within sheets of eosinophils and Charcot–Leyden crystals. Fungi are frequently detected due to their unique ability to absorb silver because of their characteristic cell walls. This is the basis of various silver stains, such as Grocott's or Gomori's methamine silver stain, which stain fungi black or dark-brown. Unfortunately, silver-based stains have high specificity but low sensitivity. A more sensitive method for identification of fungi has recently been developed that makes use of a fluorescein-labeled chitin-specific binding protein. Taylor et al (2002) used this technique to identify fungal hyphae in the vast majority of sinus mucus samples obtained from patients with CRS, even though most of these patients lacked the other classic features of

AFRS. This has become one of the major tenets of the hypothesis associated with the concept of eosinophilic fungal rhinosinusitis.

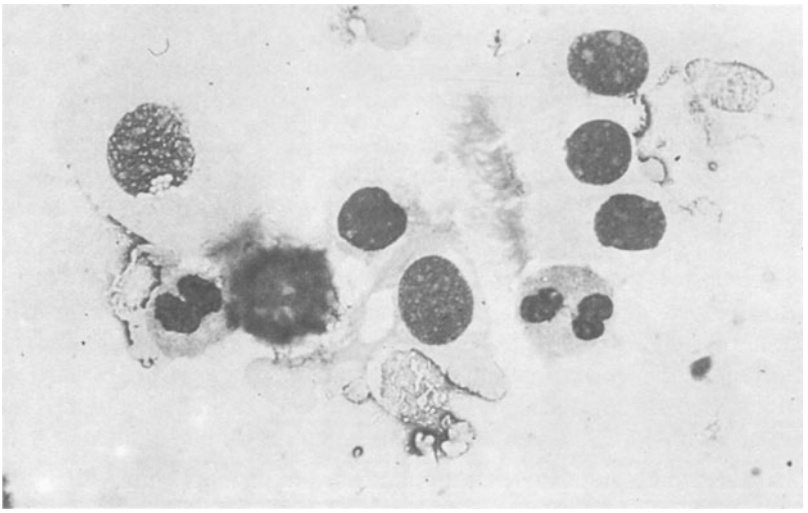


Fig. 7.13. Middle meatal lavage cytology in AFRS patient showing obvious eosinophilia and some ciliated nasal columnar epithelial cells (May-Grunwald Giemsa stain x 1000)

Fungal cultures

Fungal cultures from allergic fungal mucin might provide supportive evidence for the diagnosis and subsequent treatment of AFRS but must be interpreted with caution. It is important to realize that the diagnosis of AFRS is neither established nor eliminated on the basis of the results of these cultures. The variable yield of fungal cultures (64% to 100%) renders AFRS in the presence of a negative fungal culture quite possible (Manning and Holman, 1998). Conversely, a positive fungal culture fails to confirm the diagnosis of AFRS because it might merely represent the presence of saprophytic fungal growth. For this reason, the histological appearance of allergic mucin remains the most reliable indicator of AFRS.

DIAGNOSTIC CRITERIA

The wide range of clinical, radiographic, and immunological features that help to define the disease have been used to develop a number of diagnostic criteria (Loury *et al.*, 1993; Bent and Kuhn 1994). Patients with AFRS uniformly show five characteristics: gross production of eosinophilic mucin containing non-invasive fungal hyphae, nasal polyposis, characteristic radiographic findings, immunocompetence, and allergy to fungi (Bent and Kuhn, 1994). Based on the current literature, the diagnosis of AFRS is minimally dependent on the combination of histological evidence of fungal hyphae within eosinophilic mucin and a host allergy to that fungus. AFRS may be suspected from physical

examination or radiographic findings; but in most cases the diagnosis cannot be confirmed until sinus tissue and mucus obtained during sinus surgery have been reviewed. At the time of surgery, the patient might have a persistently opacified sinus cavity, and eosinophilic mucus and polypoid tissue might account for this opacification. Patients nearly always have type I allergic sensitivity to fungal antigens. Because of these distinctive features and distinctive complications such as bony erosion and facial dysmorphia, AFRS represents a distinct subset from the much broader group of patients with CRS.

NON–IGE-MEDIATED EOSINOPHILIC FUNGAL INFLAMMATION (EOSINOPHILIC FUNGAL RHINOSINUSITIS)

In 1999 Ponikau and co-workers suggested that colonizing fungi in sinus mucus had a much broader role in the pathogenesis of CRS, and an ultra-sensitive culture technique was used to show that 93% of 101 consecutive patients with CRS gave positive fungal cultures from nasal lavage. Examination of surgically obtained specimens from these patients also showed eosinophils and fungal hyphae in the sinus mucus of nearly all patients. It the same time it was shown that 100% of a group of healthy control subjects gave positive fungal cultures from nasal lavage. Conventional IgE-mediated allergy to fungi was not consistently observed in the patients with CRS. Ponikau *et al.* (1999) proposed that virtually all cases of CRS were associated with sensitization to colonizing fungi, and that the term allergic fungal rhinosinusitis should be replaced with eosinophilic fungal rhinosinusitis.

These suggestions were largely based on two observations, Firstly, features commonly found in chronic rhinosinusitis, such as CT or radiograph observed mucosal changes or polyposis in the nasal and paranasal sinuses, the absence of immunodeficiency, and the exclusion of invasive fungal disease, were not specific for AFS. Secondly, although the areas of hyperattenuation on CT scans are thought to be representative of the eosinophilic mucin containing fungi (Zinreich *et al.*, 1988), this feature might not be as obvious in patients who have a smaller fungal and eosinophilic mucin load.

The remaining diagnostic criteria for AFS were thought to be more disease-specific: (1) the presence of fungi, (2) the presence of allergic mucus containing clusters of eosinophils with Charcot-Leyden crystals, and (3) the existence of an IgE-mediated allergy Bent and Kuhn (1994). Nevertheless, when newer findings are considered, these three last criteria cannot be seen as specific for AFS.

Presence of Fungi

Previously, the presence of fungi in the mucus of the nasal cavity or paranasal sinuses was a criterion required to diagnose AFS (Bent and Kuhn, 1994; Deshazo and Swain, 1995). This criterion has been difficult to meet, and several detection methods and culture techniques have been used for many years,

resulting in varying rates of fungal detection. Even when fungi could be demonstrated in a histological specimen, the cultures was often negative (Cody *et al.*, 1994; Morpeth *et al.*, 1996). Novel collection (nasal lavages instead of standard swab techniques) and culturing techniques have shown that mucus specimens from 96% of chronic rhinosinusitis patients were positive for fungi, and most demonstrated the presence of multiple organisms. For the first time, specimens from healthy controls were also cultured, demonstrating the presence of fungi in 100% of the specimens.

In order to improve the sensitivity of fungal culture techniques it is necessary to collect an adequate amount of mucus. Rapid transportation and processing in specialized mycological laboratory is a prerequisite, and mucolytic agents can be used to chemically break the disulfide bridges in the mucin to release the entrapped fungi. This procedure liquefied the mucus and allowed direct contact of the fungi with the culture media, and fungal recovery was improved by using various culture media and a range of incubation temperatures. Subsequently, a novel chitin-based staining technique was developed that could demonstrated the presence of fungi in the mucus of 100% of surgical chronic rhinosinusitis specimens. This has emphasized the limitations and the lack of specificity of previous staining techniques (Taylor *et al.*, 2002).

Presence of Eosinophilic (Allergic) Mucus

Originally, the term allergic mucus was based on the historic association of eosinophilia and IgE-mediated allergy. It is now recognized that the so-called allergic mucus occurs without any detectable IgE-mediated allergy. Thus, the terminology has been changed to the more descriptive eosinophilic mucus (Kupferberg *et al.*, 1997).

Studies where extra care has been taken to ensure the preservation of mucus have histologically detected eosinophilic mucus, containing clusters of eosinophils, in 96% and 94% of consecutive surgical chronic rhinosinusitis patients (Ponikau *et al.*, 1999; Braun *et al.*, 2003). This abundance of eosinophils in the mucus of chronic rhinosinusitis patients suggests that tissue eosinophils in chronic rhinosinusitis are only in transit through the mucosa and migrate into the mucus to form characteristic clusters. Interestingly, this cluster formation seems to occur around fungal elements, suggesting that eosinophils target fungi in the mucin of chronic rhinosinusitis patients and release their toxic granule proteins, such as the major basic protein, onto the organisms (Ponikau *et al.*, 1999; Braun *et al.*, 2003).

RECENT CONCEPTS IN ALLERGIC FUNGAL SINUSITIS

Fungal culture in chronic rhinosinusitis

Previously, the presence of fungi in the mucus of the nasal cavity or paranasal sinuses was required in order to diagnose AFS (Deshazo and Swain

1995; Schubert and Goetz, 1998). Nevertheless, to prove the presence of fungi has been difficult – several detection methods and culture techniques have been used for many years, resulting in varying rates of fungal detection (Collins *et al.*, 2005).

Fungi in CRS patients are found mainly at sites where mucus is present. Recently, striking progress has been made in the development of better techniques to detect fungi in nasal secretions. The technique chosen as a method to sample the nose is important with respect to the excepted yield. Iirrigation techniques allow sampling of a large amount of mucus in comparison to swab or brush techniques. This can be seen clearly in Jiang *et al.*'s (2005) study, with a higher culture rate in lavage specimens (49%) as compared to middle meatus swab specimens (11.8%).

In a recent study by Ragab *et al.* (2006) validating the mycological culturing technique using the same collection technique as Ponikau *et al.* (1999), the authors found a 100% culture rate from the total nasal lavages of healthy subjects. A similar total culture rate was observed in CRS patients (nasal vestibule 8%, middle meatus lavage 44% and nasal cavity lavage 36%) when irrigation suction was used for specific areas in a sequential order under endoscopic guidance, (Ragab *et al.*, 2006).

In fungal culture, the presence of at least one viable spore per specimen is theoretically sufficient for a positive result. Non-viable fungus material, however, is generally not detected by culture techniques, but can be detected by other techniques, e.g. polymerase chain reaction (PCR). Both Polzehl *et al.* (2005) and Kim *et al.* (2005) have used PCR to obtain a higher fungal detection rate (44 and 92%, respectively) than obtained by culture. Granville *et al.* (2004), used histological correlation of fungal sinusitis to culture, and showed that most of the fungi obtained through culture in classic AFS were dematiacious fungi. Dematiacious fungi (87%) and species of *Aspergillus* (13%) are the fungi most commonly identified in AFS in the literature (Marple *et al.*, 2001). The lower rate of culture for dematiacious fungi and the higher culture rate for other genera seen in CRS studies may explain the erroneous diagnosis of eosinophilic fungal rhinosinusitis for all CRS patients. Fungi are ubiquitous saprophytes and many are commonly airborne and can occur in the respiratory tract as a result of inhalation (Geiser *et al.*, 2000). The retention and clearance of fungal material after inhalation depends on many factors, including physical and chemical properties of both the fungi and the mucous surfaces, the anatomical location of the deposition site, and the nature of the structures with which the particles interact at the site of deposition (Geiser *et al.*, 2000). Thus, during respiration many fungal propagules will adhere more to the mucus gel layer than to the skin of the vestibule that contains long chain fatty acids that inhibit fungal colonization (Halonen *et al.*, 1997). In addition the nose is the first mucus-lined part of the respiratory tract, and more fungi could be expected from the nasal lining than from the lower airway (Ragab *et al.*, 2006).

The immune response to fungi in chronic rhinosinusitis

The normal immune response varies with respect to the fungal species encountered. The relative importance of specific innate and adaptive defence mechanisms differs depending upon the organism and anatomical site of interaction. Within a species, the fungal morphotype (e.g. yeast, pseudohyphal and hyphal phases of *Candida albicans*) may be an important determinant of the host response. Whereas yeasts and conidia are often effectively phagocytosed, the larger size of hyphae precludes effective ingestion and requires interactions of different inflammatory cells (Shoham and Levitz, 2005).

Several shared defence mechanisms are involved in response to a range of fungi. Neutrophils, eosinophils, macrophages and monocytes are fundamentally important anti-fungal effector cells. Recent studies have shown that patients with CRS have peripheral blood T cells that proliferate and produce interleukin-5 in response to *Alternaria* (Shin *et al.*, 2004), although the fungus was found over a variable limited range (0–44%) in cultures of CRS patients. Circulating T helper 2-like cells could reflect the prevalence of *Alternaria* in the subject's environment, and the secondary development of antigen-presenting process and immune responses. Circulating *Alternaria*-specific T cells may be a relatively common and non-specific development in allergic rhinitis, asthma and eosinophilic sinusitis.

Pant *et al.* (2005) studied the fungal-specific humoral response in eosinophilic CRS. In contrast to IgE, fungal-specific IgG, IgM and IgA were present in the serum of all eosinophilic CRS patients and controls, consistent with mucosal exposure to common environmental fungi. Destruction of fungal hyphae may merely represent a non-specific feature of the activated primed eosinophils. It is also possible that any 'primed' eosinophils (e.g. Churg–Strauss vasculitis, asthma or idiopathic hypereosinophil syndrome) could function equally well in a fungicidal manner. Eosinophils lack antigen receptors, and so their ability to destroy fungal hyphae is a non-specific innate immune response or opsonization of the fungus by IgG (or IgA) in the absence of IgE (Pant *et al.*, 2005).

The histological markers for AFS are the striking numbers of tissue eosinophils in the sinuses in contrast to an absence of eosinophils in the sinuses of healthy controls (Manning *et al.*, 1998; Shin, 2001). Khan *et al.* (2000) also showed the presence of numerous intact or degenerated eosinophils (eosinophils concretion) in the sinus contents (mucin) of AFS patients. In a study by Ragab *et al.* (2005) , eosinophilia was identified in only 33.6% of the positive fungal middle meatal culture cases. Watanabe *et al.* (2004) used transmission electron microscopy to study the nasal discharge from five patients with AFS. They showed that fungal hyphae surrounded by eosinophils in only one patient, although fungal hyphae were detected in the mucus of all five. This is similar to the absence of eosinophilia in 66.4% of positive middle meatal lavage fungal culture in the study by Ragab *et al.* (2005). Watanabe *et al.* (2004) showed that in

a few cases eosinophils phagocytosed the cuticle substance of the hyphae into a sheet-like invaginated space and released granular protein into that space in the presence of fungal hyphae. This may be an innate immune response or the eosinophils responding *in vitro* to opsonized targets, however, the destruction of hyphae by eosinophils does not provide evidence that this is a fungal-mediated disorder.

Eosinophilia has been reported in 40% of negative middle meatal lavage culture cases (Ragab *et al.*, 2005). Eosinophil counts can be elevated by causes other than fungal aetiology, e.g. allergic as well as nonallergic syndromes, most notably non-allergic rhinitis with eosinophilia and acetylsalicylic acid triad syndrome (Ferguson, 2000), or as a result of superantigenic stimulation (Ferguson, 2000).

Relation of fungi to the clinical aspects of chronic rhinosinusitis

Ragab *et al.* (2005) reported that no correlation existed between fungal culture, cellular and other clinical parameters such as the visual analogue scale for the symptoms, nasal polyposis score or computed tomography score. Similarly Murr *et al.* (2006) in another study *et al.* using quantitative PCR and quality-of-life survey in CRS, found no significance to the presence or absence of fungi recovered, the type of fungi or the possible impact of fungi on the quality-of-life survey results.

Treatment

The analogy of AFS to ABPA pathophysiology can also be extended to treatment (Schubert, 2007). There is some controversy over treatment but as the fungi involved are antigenic and not infectious then successful treatment depends on three steps (see Table 7.10).

Table 7.10. Treatment of AFRS

Surgically debriding the sinuses of fungal antigens, allergic mucus and irreversibly diseased tissues.
Preventing recurrent fungal growth or colonisation.
Modifying the pathological immune response.

Surgical treatment

The main aspect of treatment is surgical intervention with three particular goals (Marple, 1999). Firstly the surgery should completely remove accumulated allergic mucin and fungal debris from the sinuses involved, and so attenuate the continuous antigenic stimulation and disrupt the underlying immunologically mediated inflammatory process. Secondly surgery should provide permanent

drainage while preserving the integrity of the paranasal sinus mucosa. Thirdly, surgery should provide postoperative access to the previously diseased areas.

Allergic mucin fills the cavity with more significant inflammation and polyps located at the sinus ostium that extend into the infundibulum, middle meatus and sphenoethmoidal recess. Recognition of this allow surgeon to follow polyps to the disease. Also, the expansible behaviour of the disease allows increased access to the paranasal sinuses with widening of the pre-chamber regions (Ryan *et al.*, 2007).

Corticosteroids

Systemic corticosteroid therapy was successful in the treatment of ABPA and it has become a standard treatment for AFRS, with the main aim of modifying the immune response. The potent anti-inflammatory and immunomodulatory effects have made corticosteroids the mainstay in the management of AFS.

Kuhn and Javer (2000) showed a significant increase in the time to revision sinus surgery in patients who received prolonged courses of postoperative corticosteroids. Length of treatment and dose are still controversial. Kuhn *et al.* (2000) used the following protocol with 4 years follows up for 11 AFRS patient and they demonstrated reduction in mucosal stage and IgE level postoperatively while the patient remains in systemic corticosteroids therapy:

Within 48 h of surgery: 0.4mg/kg/day for four days, 0.3mg/kg/day for four days, 0.2mg/kg/day or 20 mg/kg whichever is greater for one month.

One month post-op: 0.2mg/kg/day until the mucosal stage returns to stage 0 for four months.

After four months: 0.1mg/kg/day for two more months at stage 0. Start nasal steroid powder spray at 1 spray in each nostril three times daily.

After six months at stage 0: continue nasal steroid powder spray for one year.

Using this protocol 8 out of 11 patient had a recurrence of the disease with average time of recurrence being 10.6 months (range 2 to 27 months).

Topical corticosteroids are accepted as the standard postoperative therapy but are not used preoperatively because of limited delivery of local corticosteroids to the sinuses (Schubert, 2007). Kuhn and Javer (2000) suggested using topical corticosteroids at triple the dose used for allergic rhinitis.

Antifungal drugs

Antifungal drugs are used in order to modify the fungal growth or colonization. Systemic antifungals were initially used to control the potential progression to invasive forms. Denning *et al.* (1991) studied the effects of 200mg Itraconazol twice daily for 1 to 6 months in patients with ABPA and showed a

decrease in both total IgE and systemic corticosteroid use. Rains and Mineck (2003) reported successfully treating 139 AFS patients postoperatively with oral high-dose Itraconazole and topical and oral corticosteroids with a reduction in the return-to-surgery rate. Other anecdotal reports have also reported significant positive responses to oral Itraconazole, but questions arise as to whether this could be caused by its steroid anti-inflammatory effect as opposed to its antifungal action. Voriconazole is less toxic than other antifungal drugs and may give promising results.

There are a number of disadvantages to using systemic antifungal drugs. AFRS is a non-life threatening disease and the cost of therapy is high. Serious side effects may occur and Amphotericin B requires intravenous access and frequent premedications, while liver function should be followed with Ketoconazol. The fungi are present extarmucosal (outside the range of drug circulation), and so a systemic antifungal must be excreted extramucosaly.

Local antifungals may provide some control of recurrence. Bent and Kuhn, (1996) studied the *in vitro* response to ketoconazol, amphotericin B, itraconazol, nystatin, and fluconazol of 22 fungi obtained from 15 patients with AFS. They determined that the minimal inhibitory concentrations could be exceeded using topical delivery method. They proposed the initiation of 1 mg/ml of 0.125% topical ketoconazol (dissolved in acetic acid solution). In a separate study 16 patients with a history of allergic fungal sinusitis were given fluconazole nasal spray and followed for three months. A stabilization or improvement of the disease was seen in 12 of the 16 patients who were treated with this protocol without significant side effects (Jen *et al.*, 2004). It is not clear whether the improvements seen in this study were due to corticosteroids or antifungal drugs. Concentrations of 200 and 300 μg/mL successfully prevented fungi growth at the conclusion of the study (Shirazi *et al.*, 2007). The current concentration of commercially available topical amphotericin B (100 μg/mL) seems ineffective in eradicating fungi *in vitro*. Prior reports of antifungal therapies in CRS have yielded mixed results.

Ponikau *et al.* (2005) reported positive results in an open label pilot study using topical amphotericin and also in a more recent randomized double-blind study of the same medication. However, another randomized double-blind study of topical amphotericin-B nasal spray in CRS from Europe reported significant worsening of symptom scores in the active treatment group and no significant difference in CT scores between the treated and control group. A concentration of 100 μg/ml twice daily for 3 months did not reduce clinical signs and symptoms in patients with CRS (Ebbens *et al.*, 2006).

Additional issues associated with the use of amphotericin B are its high cost, poor stability in solution, and the fact that it cannot be administered in saline solution. It has also been noted to be cytotoxic to the nasal epithelium, and this has been hypothesized as one reason that it could have a therapeutic effect in some patients (Weschta *et al.*, 2004). Kennedy *et al.* (1997) used high-dose terbinafine over a period of 6 weeks in a double-blind, multi-centre, placebo

controlled study but failed to show a significant improvement over placebo (Jornot *et al.*, 2003). Both CT scores and subjective symptomatology were similar in the active treatment and control group at the conclusion of the study.

Specific immunotherapy

The idea of using immunotherapy comes from the similarity between AFRS and ABPA. However there are empirical and theoretical concerns that giving immunotherapy with fungal antigens to patients with either AFRS or ABPA might incite further allergic reactions by adding to the fungal antigenic stimulus. In AFRS surgery can remove the fungal material and antigenic stimulus from the sinuses, and so it has recently been postulated that immunotherapy could be beneficial rather than harmful (Marple, 1999).

Other than the Mabry *et al.* (1998) series for immunotherapy. There is little published information about immunotherapy. Ferguson (2000) reported on the immunotherapy treatment of seven AFRS patients but no improvements were noted. This may be because the patients received the immunotherapy before surgical treatment (Ryan and Marple, 2007).

One effect of immunotherapy is an increased synthesis of antigen specific IgG, which is thought to block the IgE, mediated type 1 reaction responsible for atopy. Previously it was difficult to determine which type of antigens to use for immunotherapy, but this may be clearer following the discovery of an 18KD allergen which has been suggested as a pan-allergen for fungi (Chrzanowski *et al.*, 1997). Another study by the same authors compared two groups of patient with AFRS treated with the same regimen except for immunotherapy. The results showed that for the patients receiving immunotherapy there were statistically significant differences in the quality of life scores, endoscopic mucosal staging and no requirement for courses of systemic corticosteroids (Mabry *et al.*, 1998).

Other methods for assisting treatment

Environmental controls may assist in treatment. Noble *et al.* (2001) focused on the patient environment and the possibility that patients cured of AFRS could return to their original environment and be re-inoculated. Metal surfaces, air filters and insulation materials present in residential and commercial building can serve as foci of fungal growth and the dissemination of airborne conidia. Noble *et al.* (2001) also showed that 15 of the predominant fungi recovered from the air samples of selected patient residences included the same species as had been recovered from their mucin.

Nasal sinus saline lavages may also be helpful as an adjuvant treatment to promote mucociliary clearance and promote clearance of fungal load (Schubert, 2007).

Follow up

Each patient can be assessed by – Serological markers (IgE level)- Prednisone dose and the clinical stages are plotted monthly on a graph. They are assisted monthly for 6 months and then bimonthly for 3-5 years. The patients need to be followed for up to 1 year after the prednisone therapy (Kuhn and Javer, 2000).

Prognosis

All patients need FESS (Functional Endoscopic Sinus Surgery) with regular frequent endoscopic follow up. Patients who received postoperative steroids had less endoscopic confirmed disease (Kuhn and Javer, 2000). The length of time before any recurrence is dependent on the amount of fungi and fungal antigen remaining after FESS, and individual variations in type 1 immunity. A staging system provides a framework for the clinician to observe responses to medical therapy and to assess the need for further surgery. The short-term prognosis for AFS is good but the patient and physician should understand that it will require continuing treatment and that it appears to be a chronic disease (Kuhn and Javer, 2000).

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CHAPTER - 8

BIODIVERSITY OF FILAMENTOUS FUNGI ON SOILS AND SANDS

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Introduction

The diversity of soil organisms is more extensive than in any other existing environment as far as the living forms are considered. The soil biota contains representatives of all groups of microorganisms (fungi, bacteria, algae and viruses), as well as microfauna, such as protozoa and nematodes (Campbell and Puri, 2002).

No complete inventory of soil fungi has been produced for a single geographic region, but numerous intensive and a few extensive surveys have been carried out in Europe, North America, The Middle East, India, Japan, Taiwan, Australia, New Zealand, and The Artic (Bills *et al.*, 2004).

Soil fungi species are included in the compilations of different authors cited by Brigde and Spooner (2001): Gilman (1957), Barron (1968), Domsch *et al.*, (1993) and Watanabe (1994). The numbers ranged from 700 to 1200 species.

In 2001, The Centraalbureau Voor Schimmelcultures (CBS) preserved 2210 species of soil fungi, about 70% of the known species available in culture. The current estimation for described culturable soil fungi is approximately 3150 species. If, in addition, we consider the ca. 150 spp. of nonculturable *Glomerales,* then the number of soil fungi results in the 3300 species currently known (Gams, 2007).

In what concerns macrofungi, they comprise 10% of the total fungal diversity (Rossman, 1994; Mueller *et al.*, 2007). The last study made by Mueller *et al.* (2007) gives an estimation of 53 000-110 000 macrofungi, most of them being *Ascomycota* or *Basidiomycota.*

The type of vegetation and geographic aspects are factors which affect the species richness and the composition of macrofungi on each site (Lodge *et al.*, 2004).

In general the success of fungi to reach and colonize a patch of soil is mainly due to their competitive saprophytic ability, expressed by fast mycelial growth, spores production, possession of an efficient and extensive system of powerful enzymes and tolerance to antibiotics, salinity, heavy metals, fungicides and temperature.

The diversity of soil fungi depends on the chemistry, texture and water holding capacity of the soil. Physical factors such as temperature, pH, organic matter, also have a great influence on the microbial life.

Soil temperature affects all chemical reaction rates; therefore micro-organisms have little control over how fast their own metabolic processes take place.

The soil pH is a critical environmental factor, because it has direct effect on whether or not the microorganisms can survive and grow. Fungi are more tolerant to acidic pH than soil bacteria and actinomycetes who are more tolerant to alkaline conditions than other organisms (Coyne, 1999).

From the above mentioned facts it can be considered that soil fungal similarity is an outstanding indicator of environmental similarity (Bills *et al.*, 2004).

Sand fungi

Coastal sand dunes constitute environments with a complex dynamics where plant colonization exhibits a diversity of Arbuscular Mycorrhizal fungi (AM). Nicolson (1959) cited by Beena *et al.* (2001), recognized for the first time the importance of AM on the growth and development of dune plants. The major benefits for the sand dune plant species due to AM fungal associations are an increase in nutrient supply and salinity tolerance, a reduced abiotic stress and the formation of wind resistant aggregates (Beena *et al.*, 2001).

The fungi forming AM were placed in the order Glomales of Zygomycetes (Carlile *et al.*, 2001), and, more recently, in a new Phylum, *Glomeromycota* (Schüßler *et al.*, 2001), taking into account molecular, morphological and ecological aspects.

While the coastal temperate sand dunes have been extensively surveyed for the occurrence of arbuscular mycorrhizal fungi, the studies for the subtropical and tropical regions are scarce (Alarcón and Cuenca, 2005).

In the sand dunes a large variety of macrofungi can also be found, the majority being saprobes or mycorrhizal symbiontes although some may also be pathogens of plants and fungi (Mueller *et al.*, 2007) or animals (Lodge *et al.*, 2004).

Macrofungi studies are very important to provide more information about their diversity, distribution, specific levels of endemism, and also to know the threatened species. For example, in southern Europe, some species of macrofungi (*Agaricus devoniensis, Gyrophragmium dunalii, Conocybe dunensis*, *Pleurotus eryngii* and *Peziza ammophila*) are declining and severely threatened due to human activities, namely tourism and constructions (Senn-Irlet *et al.*, 2007).

Concerning the sand beaches, important data can be found in the Guidelines for safe recreational water environments (WHO, 2003). Among these data are the studies conducted by Soussa in 1990 that resulted in the isolation of dermatophytes in Portuguese beaches, the most common being *Trichophyton mentagrophytes* and *T. rubrum,* and *Microsporum nanum*, all isolated from non-flooded sandy areas with organic residues; it is also referred a survey conducted by Izquierdo *et al.* (1986) in which beach sands along the Mediterranean coast of Spain were analysed, resulting in the isolation of 16 species of fungi, most of them being *Aspergillus*, *Penicillium* and *Cladosporium*.

In another Spanish study from Roses Codinachs *et al.* (1988) the most frequently isolated genera were *Penicillium, Aspergillus, Cladosporium, Alternaria, Mucor*, *Monilia, Cephalosporium*, *Verticillium* and *Chrysosporium*; again in the Mediterranean beaches of Spain, the most commonly isolated genera were *Penicillium, Cladosporium, Aspergillus, Acremonium, Alternaria* and *Fusarium* (Larrondo and Calvo, 1989).

The Egyptian sandy beaches were studied in 36 samples collected in nine locations and the most frequent fungi genera were *Penicillium*, *Aspergillus*, *Chaetomium* and *Trichoderma* (Migahed, 2003).

Anamorphic fungi, Ascomycota and Zygomycota have been reported as the most common fungi in coastal sandy beaches of California, Florida and Hawai (Dabrowa *et al.*, 1964; Bergen, Kishimoto and Baker, 1969 and Wagner – Merner, 1977), cited by Migahed (2003).

The evaluation of the microbiologic quality of Portuguese sand and water beaches has been improved in the last decade, as the quality of environmental aspects of beaches affects human health besides being crucial for tourist selection.

In the Portuguese sandy beaches of Fonte da Telha and Carcavelos, São José *et al.* (1994) isolated and identified a large number of genera: *Acremonium, Alternaria, Arthrobotrys, Aspergillus, Aureobasidium, Beauveria, Chrysosporium, Cladosporium, Epicoccum, Fusarium, Geotrichum, Humicola, Nectria, Nocardia, Oedemium, Paecilomyces, Penicillium, Sporothrix, Staphylotrichum, Stemphylium, Trichosporon, Trichothecium* and *Verticillium.* In another study, Mendes *et al.* (1998) surveyed the coastal areas of Lisbon and Tagus river valley

beaches, for the evaluation of the mycological quality of the sands, based on the detection of keratinophilic fungi, yeasts, and potential pathogenic, allergenic and/or environmental fungi. The results showed a satisfactory to good quality for these sandy beaches and indicated the allergenic and environmental fungi as the most common in these environments.

The following table, based on reports of microbiological quality of sands of the Portuguese coastal beaches (Associação Bandeira Azul da Europa, 2002) indicates the *taxa* that may be found:

Zygomycota	Ascomycota	Anamorphic fungi	
Absidia sp. ▲	*Chaetomium* sp. ▲	*Acremonium* sp. ▲	*Monilia* sp. ▲
Cunninghamella sp. ▲		*Alternaria* sp. ▲	*Ochroconis* sp. ▲
Mucor sp. ▲		*Aspergillus* spp. ●	*Paecilomyces* sp.
Rhizopus sp. ▲		*Aureobasidium* sp. ▲	*Penicillium* sp. ▲
		Beauveria sp. ▲	*Phialophora* sp. ▲
		Botrytis sp. ▲	*Phoma* sp. ▲
		Cladosporium sp. ▲	*Scopulariopsis* sp. ●
		Chrysosporium sp. ●	*Scytalidium* sp. ●
		Curvularia sp. ▲ *Drecheslera* sp. ▲	*Scedosporium* sp. ●
		Epicoccum sp. ▲	*Stachybotrys* sp. ▲
		Geotrichum sp. ▲	*Stemphylium* sp. ▲
		Gliocladium sp. ▲	*Trichoderma* sp. ▲
		Fusarium sp. ●	*Tricophyton* sp. ■
		Microsporum sp. ■	*Ulocladium* sp. ▲
			Verticillium sp. ▲

● Filamentous fungi potentially pathogenic ■ Dermatophytes ▲ Environmental fungi

Most isolates are anamorphic, potential pathogens or environmental fungi and two of them are dermatophytes (Associação Bandeira Azul da Europa, 2002).

The last official results relative to the surveyed sands were published by Associação Bandeira Azul da Europa (2007). This report also presents the adopted methodologies, lists the pathogenic agents to be surveyed and the parameters to be considered. The fungi to be surveyed are distributed by three categories, listed below:

I - Yeasts: *Candida albicans, Candida, Cryptococus neoformans,* and other yeasts

II - Filamentous fungi, potentially pathogenic and/or allergenic: *Aspergillus fumigatus, Aspergillus niger, Aspergillus, Chrysosporium, Fusarium, Scytalidium, Scedosporium, Scopulariopsis, Histoplasma, Coccidioides, Exophiala, Fonsecae, Phialophora* and others

III- Dermathophytes: *Trichophyton, Microsporum, Epidermophyton.*

The maximum recommended values (MRV) and the maximum acceptable values (MAV) indicated for each category, in cfu/g (colony forming units per gram of sand) are presented in the following table:

Category	I	II	III
MRV	3	5	1
MAV	60	85	15

A reduction in the isolation of dermatophytes as well as saprotrophic fungi has been verified in recent years throughout the Portuguese coast in the summertime by Associação Bandeira Azul da Europa (2002, 2007).

Current methods for sampling and isolation of fungi

The soil fungi are primarily found in the top 10 cm of the soil, and are seldom found below 30 cm (Atlas and Bartha, 1998). They have the ability to remain in the form of resting organs (sclerotia, oospores, chlamydospores and other forms) or as an active or inactive mycelium (Davet and Rouxel, 2000). So the soil samples must be collected from the upper surface of the mineral soil or its equivalent, after careful removal of the litter and humus. They must be representative of the site, and so the composite samples are obtained by mixing equal amounts of material taken from soil samples collected over a wide area (Pepper *et al.,* 2000).

A technique for qualitative assessment of the spatial relationships among soil microorganisms, and among them filamentous fungi, is the contact slide or buried-slide of Cholodny-Rossi. It is also used to illustrate the orientations of soil microorganisms, among them and relative to soil particles (Pepper *et al.,* 1995). The exposure of electron microscope grids in natural environments and the use of pedoscopes are variations of this technique (Atlas and Bartha, 1998).

Methods of isolating fungi can be divided into direct and indirect methods, most of them well described (Bills *et al.,* 2004).

Direct methods are very useful if the fungus produces clearly visible spores on the surface of the substratum. When using direct methods the observed spores or mycelia are transferred to a sterile nutrient culture medium. Indirect methods consist in the incubation of a substratum sample in a sterile nutrient medium where, usually, numerous colonies will be produced. These colonies can be transferred to new culture media to obtain pure cultures.

There are several indirect methods for the isolation of soil fungi, the plating of serial dilutions of soil suspensions being the most frequently used. This is a simple and quick method presenting reasonably repeatable results, thus yielding excellent data (Bills *et al.*, 2004).

Warcup method consists of dispersing minute quantities of finely pulverised soil throughout the bottom of a sterile Petri dish. Cooled agar is then poured over

the soil and the plates are agitated gently to disperse particles. This method is simple and good for preliminary or quick assessment of soil species; however, young colonies embedded in agar are more difficult to remove (Bills *et al.*, 2004).

The particle filtration technique, also known as soil washing, consists in the addition of distilled sterilized water to an Erlenmeyer flask with a sample of soil. The flask is shaken in order to assure an efficient yet smooth washing of the sample and, after washing, supernatant aliquots are taken and subject to filtration. The membranes thus obtained are placed on Petri dishes containing selective media for fungi search (Gams and Domsch, 1960; Associação Bandeira Azul da Europa, 2002; Bills *et al.*, 2004). This method favours the isolation of mycelia fragments immersed in the substratum while reducing the recovery of colonies initiated from spores. Also the isolation of species with chlamydospores embedded in organic particles may be favoured because most probably their propagules are not entirely removed by the soil washing.

Another technique to isolate soil fungi uses Scotch tape, to take successive samples from an exposed soil surface. The samples are transferred to a selective agar medium and incubated. The position of colonies on the plate reveals the location and distribution of the original fungi in the soil. This technique can be combined with replica plating to test the colonies in different media, thus contributing to their identification. Nevertheless, colonies can grow in juxtaposition, making isolation difficult if the medium does not contain a dye (Rose Bengal) that may control the growth rate of the faster growing fungi.

There are still some other indirect isolation procedures: Drechesher's method, soil swabbing and stamping method, and baiting method.

The Drechesher's method is based upon sprinkling a few grams of fresh soil directly onto a medium supplemented by antibiotics. The Petri dishes are colonised by soil fungi species after a few days of incubation. The conidia or sporangiospores of moist-spored species can be touched with a fine needle and transferred to agar slants, while the dry-spored species are transferred with a needle moistened with sterile glycerol (Bills *et al.*, 2004). The swabbing and stamping method can be applied to any soil, preferably when the propagule density is low, as in deserts (Bills *et al.*, 2004).

The baiting method consists in the baiting of the fungi with the help of a living or inert substratum and then isolating it from the substratum when the life cycle allows (Davet and Rouxel, 2000). The success of this technique depends on the type and selectivity of the bait, as well as the environment in which the relationship between this bait and the soil mycota is established.

Concerning the isolation of sand fungi, particle filtration and hair baiting or To-Ka-Va are very often used methods. To-Ka-Va is specific for the isolation of keratinophylic fungi. It consists in the spreading of sterilized small fragments of child hair over the surface of samples of sand distributed in Petri dishes. The sand must be moistened with distilled sterilized water and incubated for 2

months. Observations are carried out under the stereomicroscope on a weekly basis for all that time. Once the growth of fungi over the hair is observed, the mycelium or the colonized hair fragment itself is inoculated in selective culture medium (Benedek, 1962 ; São José *et al.*, 1994).

Current methods of identification of fungi

The most common means of identifying fungi is based on the characterization of spores (conidia zygospores, ascospores and basidiospores) and reproductive structures (conidiogenous cells, conidiophores, *asci* and *basidia*), usually by the use of dichotomous keys.

For the identification of anamorphic fungi species it is important to complement the morphological characteristics of the spores (according to Saccardo classification) with the ontogeny (Kirk *et al.*, 2001).

The use of different taxonomy keys and the comparison of characteristics observed are vital to achieve a correct identification of the fungi. With the material mounted in lactophenol or lactophenol with cotton blue, micro-photographs of the reproductive structures are taken for identification purposes, using a light microscope.

After the isolation of filamentous fungi, the characterization of their macro and micro morphology for the identification of the genera and species is carried out with the help of previously selected pictorial keys.

The macroscopic characterization of the colonies is based on the observation of colour, rate of growth, border, texture, relief and the diffusion of pigment in the medium (Sidrim and Moreira, 1999).

The reproductive structures from the anamorphic fungi are very delicate and are easily broken, so for obtaining good preparations, the selected fungus is inoculated in a sterile agar block placed on a sterile microscope slide. The block is covered with a sterile cover glass and the whole set-up is incubated in a sterile Petri dish at 20-25°C. The intact somatic and reproductive growth is directly examined and used for microscope characterization and identification. The fluid mounting is lactophenol with or without cotton blue. Using lactophenol with cotton blue, the hyaline cytoplasmatic structures are easily and better observed. This methodology is known by slide culture technique or Riddell Method (Riddell, 1950; Kirk *et al.*, 2001).

For each fungus of the *Phyla* Zygomycota and Ascomycota, and for the group of anamorphic fungi, the dimensions of the principal diagnosing reproductive structures are measured under a dissecting microscope. The examination and measurement of spores (zygospores, ascospores and conidia) must be done in mature structures.

For good microscope preparations of *Aspergillus*, *Penicillium* and other fungi producing many hydrophobic spores, a drop of alcohol is added to wash away the mass of spores, before the addition of the mounting medium.

Statistical analyses (diversity and similarity indices)

After the identification of fungi the following values may be determined:

- Number of colonies forming units (C.F.U.) of each fungal taxa = number of the colonies of one fungus (Madigan and Martinko, 2006);
- Frequency of occurrence (F.O.) of all taxa = number of colonies of one fungus divided by the total number of colonies (Tan *et al.*, 1989);
- Richness (S) = number of taxa (species) associated with a particular sample /area / habitat /substratum (Zak *et al.*, 2004);
- Shannon index (H') = $-\sum_i^s$ = pi.Ln (pi), where **pi** is the proportion of individuals of the species **i**, that contribute to total diversity (Maria and Sridhar, 2002);
- Evenness (E) = H'/ H'_{max} where H'_{max} is the maximum value of diversity for the number of species that are present (Maria and Sridhar, 2002);
- Sorenson index (C_s) = 2j/(a+b), where **j** is the number of taxa common to both sites, **a** being the number of taxa in site A and **b** being the number of taxa in site B. This index is equal to 1 in case of complete similarity and 0 if the sites are dissimilar with no species in common (Abdel-Wahab and El-Sharouny, 2002).

Molecular approaches

Several molecular approaches have been used during the last decades in order to assess genetic diversity, at the level of gene expression (based on polypeptides and RNA molecules) or at the level of the genome itself (nuclear and mitochondrial DNA).

Rodriguez *et al.* (2004) referred these approaches and did a comprehensive description and discussion of the methods applied to the study of fungal diversity and taxonomy. Summarizing, the diversity of the nuclear genomes is mainly addressed by approaches based on restriction analysis (Restriction Fragment Length Polymorphisms – RFLPs), amplification by Polymerase Chain Reaction (PCR) and sequencing. PCR alone or in combination with other techniques is the preferred one, mainly because it can be applied to samples with very small amounts of nucleic acids. PCR may be performed either by using specific primers designed for the amplification of particular regions or by Arbitrarily primed PCR (ApPCR), in order to obtain Random Amplified Polymorphic DNA (RAPD). Moreover, after the identification of diagnosing polymorphic markers, they can be cloned, sequenced and used to design marker-specific PCR primers for dual-primer PCR (dpPCR) analysis. The product of this reaction may be amplified with a new set of primers that anneal within this region. This process is designed by nested-primer PCR (npPCR) and results in an increased sensitivity, allowing the detection of few genomes in any complex sample.

Specific primers for conserved regions of β-tubulin and ribosomal RNA (rRNA) genes, namely for the small and large sub-units of rRNA and for the internal transcribed spacer (ITS), have been used with consistent results, by putting together PCR amplification and sequencing of the PCR products.

RNA molecules may also be analysed by sequencing, by Reverse-Transcription-coupled PCR (RT-PCR) and by Real-time PCR which is a method of quantitative transcript analysis. These approaches are more often used when studying fungal biomass and physiological activities of fungi *in situ.*

Mitocondrial DNA (mtDNA) can also be used to study fungal biodiversity and taxonomy as well as the role of fungi in ecosystem dynamics. The smaller size of mitochondrial genome relative to nuclear genome contributes to the choice of this approach. The ease of mitochondrial DNA purification, its high copy number, the lack of methylated bases, and haploid nature (which eliminates genetic recombination), as well as the existence of RFLPs and availability of known gene sequences for PCR amplification, are among the reasons cited to advocate its utilization. Obviously, the above mentioned methods also apply to the study of these genomes.

Experimental

The isolation and characterization of fungi from Portuguese soils and sands, presented in this chapter as case studies, were carried out by Azevedo (2003) and Figueira (2004). Pinewoods and vineyards were chosen because they are common habitats in Portugal. The sands of two beaches were studied because they are also a common environment, as this country has a large coastal area.

Locations

The study sites for soil fungi were Mata dos Medos (Pinhal do Rei), a pinewood soil forest located at Costa da Caparica (38 5952 N -09 19'47''W; UTM 29S ME7116) covering an area of approximately 338 ha, and a vineyard soil, located at Alenquer (39°08'44''N - 09°09'65''W; UTM 29S ME8533). The collections were made in March and April, 2002 (Azevedo, 2003).

The vegetation of the pinewood forest soil is dominated by *Pinus pinea* L. and includes *Pinus pinaster* Aiton and *Pinus halepensis* Mill, *Pistacia lentiscus* L., *Rhamnus alaternus* L., *Myrtus communis* L., *Olea europea* L. var. *sylvestris*, *Arbutus unedo* L. and *Juniperus phoenicea* L.. The fauna is characterized by small species of mammals, reptiles, birds, and insects (Lado, 1994).The vineyard soil was chosen from an important region of wine production, with a very fertile soil.

For the study of sand fungi, two sandy beaches on the Portuguese west coast were selected: Rainha beach (38°41`N 09°25`W; UTM 29SMC6383) and Guincho beach (38°43`N 09°28`W; UTM 29SMC5983). The collections were made between November, 2002 and March, 2003. Guincho beach, on the southern edge of Sintra Natural Park, facing the Atlantic, is exposed to strong

westerly winds that generate high waves. Rainha beach, in the bay of Cascais is smaller, not so windy and has calm waters (Figueira and Barata, 2007).

Collection of samples from different substrata (soils and sands)

The collection of the pinewood forest soil was done with sterilised tools on four locations underneath 3 pine trees, at 15 cm deep, after carefully removing the overlying litter and humus. At the laboratory, a representative sample was obtained by mixing equal quantities (30 g) of each individual sample collected at the sampling points. Identical procedure was done for the vineyard soil (Azevedo, 2003).

The sand was collected from two different zones on the beaches of Rainha and Guincho: intertidal zone (wet sand - W) and permanently dry zone (dry sand - D).

Two lines were drawn parallel to the water line, one in the wet sand and the other one in the dry sand. Both lines were divided into three identical segments with approximately ten meters each.

In each segment, a sample of sand was collected from the surface down to 2 to 3 cm of depth (Figueira, 2004).

All the collected samples, from soils and sands, were placed in polythene bags and transported in an ice chest to the laboratory.

Recording of physical parameters

The physical parameters of the soils recorded at each field visit included pH, temperature and organic matter. For the pH determination the method described by Buol *et al.* (2003) was used and the pH values were measured by a potentiometer (Hanna Instruments HI 9321).

For the determination of the organic matter the method designated loss of weight by ignition or calcination (Costa, 1999) was used.

The temperature of the soil was determined on 3 selected sampling points for each soil. For the sand, a thermometer was buried in the dry sand for 5 minutes.

Qualitative assessing of soil microorganisms (Cholodny-Rossi method)

The Cholodny-Rossi method (Cholodny, 1930; Rossi, 1936) was applied "in laboratory" for the pinewood forest and vineyard soils and only "in situ" for the pinewood forest soil.

For each "in situ" sampling points, 36 previously numerated and sterilised microscope slides were partially buried, during 8 days, leaving 2 cm of each slide above the surface. After that period of time the slides were carefully removed, by pressing them in an inclined position. They were then put into a sterilised plastic box and brought to the laboratory.

For the laboratory assays, 250 g of each soil were put into a labeled tumbler. In each tumbler, 6 sterilized microscope slides were vertically inserted, leaving 2 cm of each slide projecting above the soil surface. The tumblers were covered with plastic wrap, secured with a rubber band; the wrap was punctured several times to allow ventilation and yet preclude excessive evaporation of moisture, and was incubated at room temperature, during 8 days. Then, each slide was gently tapped on the bench top to remove most of the soil particles, and immersed in 40% acetic acid (v/v) for 3 minutes. After washing away the excess acid they were stained for 8 minutes with phenol Rose Bengal, without allowing the slide to dry. Finally each slide was gently washed to remove the excess stain, dried and examined microscopically using an oil immersion objective (Pepper *et al.*, 1995).

Isolation and culture techniques for filamentous fungi

Soil fungi

The soil fungi were isolated by the serial dilution agar plating method. The isolation media used were Rose Bengal Agar (RBA) and Czapek–Dox (CZ) supplemented with 0.05% chloramphenicol. In sterile conditions, 10 g of each soil were placed into Erlenmeyer flasks with 95 mL of 0.85% NaCl plus one drop of 0.1% tween 80, and shaken for 5 minutes in an orbital incubator at 25°C. Then, serial dilutions were performed: 100 µl of soil suspension were transferred to a sterile tube containing 900 µl of 0.85% NaCl and mixed in the vortex (dilution 10^{-1}), this procedure being repeated until the 10^{-5} dilution was reached. Finally, 100 µl from each dilution were spread over the surface of 3 Petri dishes with the selected isolation media, (RBA and CZ), and incubated in inverted position for 3 to 4 days at room temperature (25°C).

Sand fungi

For the analysis of these fungi, the sand washing method was used (Bernard and Pesando, 1989): in sterile conditions, 40g of each type of sand (W and D) were put into Erlenmeyer flasks with distilled water (20 mL in the flask containing dry sand and 10 mL in the flask containing wet sand) and the flasks were shaken in an orbital incubator at 25°C for 5 minutes. From each flask, 200 µl of supernatant samples were removed to inoculate Malt Agar with chloramphenicol (MA) and Mycobiotic Agar (MYC) media, 3 Petri dishes of each, using a bent glass rod for the distribution of the samples over the surface of the medium. The dishes were incubated at 28°C for 4 to 5 days (MA cultures) or 15 days (MYC cultures). After these periods of time the counting of the colonies was performed, as well as the purification and identification of fungi.

When non sporulating fungi were isolated, they were inoculated into water agar medium and incubated for at least one month at 28°C, in order to induce sporulation.

Identification of filamentous fungi

This work was based on the classical taxonomy and the fungi isolates were distinguished based on the microscopic characteristics of the principal diagnosing reproductive structures, as described in the Introduction of this chapter.

The identification and classification of the soil and sand fungi were made with the help of illustrated keys of Domsch *et al.* (1980), Samson *et al.* (2002), and (2) and also based on the Dictionary of the Fungi, 9th edition (Kirk *et al.*, 2001).

All soil fungi were preserved in Potato Dextrose Agar medium (PDA) and the sand fungi were preserved in Malt agar and Sabouraud agar media. All samples were stored in McCartney flasks at 4°C.

Results and discussion

Physical parameters

The mean values of soil temperature, pH and organic matter were 10°C, pH 5 and 17.24%, and 15°C, pH 6 and 8.62%, for the pinewood forest and the vineyard soils respectively. For the sands, the mean values of temperature were 17°C at Guincho beach and 18°C at Rainha beach.

Frequency of occurrence of fungi for soils and sands

The soil and sand fungi recorded and the corresponding frequencies of occurrence are listed in tables 8.1, 8.3 A, 8.3 B. In tables 8.1 and 8.3 A, they are organized by decreasing frequency of occurrence based on a sample of 623 colony forming units (c.f.u.) from soil samples (386 from the pinewood forest plus 237 from the vineyard) and 331 c.f.u. from sand samples (215 from Rainha and 116 from Guincho).

Table 8.1 Frequency of occurrence of taxa detected in soils

	Pinewood forest soil + vineyard soil		**Pinewood forest soil**		**Vineyard soil**	
Taxa	c.f.u.	F.O.%	c.f.u.	F.O.%	c.f.u.	F.O.%
Penicillium spp. (a.f.) *	176	28.25	149	38.60	27	11.39
Aspergillus spp. (a.f.) *	94	15.09	38	9.84	56	23.63
Scopulariopsis sp. (a.f.)	51	8.19	51	13.21	0	0.00
Trichoderma sp. (a.f.) *	51	8.19	41	10.62	10	4.22
Gliocladium roseum Bainier (a.f.) *	31	4.98	16	4.15	15	6.33
Verticillium sp. (a.f.) *	27	4.33	16	4.15	11	4.64
Cladosporium cladosporioides (Fresen) de Vries (a.f.)*	27	4.33	14	3.63	13	5.49

Fusarium spp. (a.f.) *	26	4.17	10	2.59	16	6.75
Rhizopus sp. (Z)	24	3.85	0	0.00	24	10.13
Stachybotrys sp. (a.f.) *	16	2.57	5	1.30	11	4.64
Botrytis cinerea Pers. (a.f.) *	14	2.25	6	1.55	8	3.38
Mucor hiemalis Wehmer (Z)	12	1.93	0	0.00	12	5.06
Myrothecium roridum Tode ex Fries (a.f.) *	11	1.77	4	1.04	7	2.95
Acremonium strictum W. Gams (a.f.) *	11	1.77	3	0.78	8	3.38
Alternaria alternata (Fr.) Keissler (a.f.)	9	1.44	0	0.00	9	3.80
Phoma sp. (a.f.) *	9	1.44	7	1.81	2	0.84
Memnoniella echinata (Rivolta) Galloway (a.f.)	6	0.96	6	1.55	0	0.00
Pestalotiopsis sp. (a.f.)	6	0.96	6	1.55	0	0.00
Torulomyces lagena Delitsch (a.f.)	5	0.80	5	1.30	0	0.00
Mortierella ramanniana (Möller) Linnen (Z)	4	0.64	4	1.04	0	0.00
Doratomyces sp. (a.f.)	4	0.64	0	0,00	4	1.69
Absidia sp. (Z)	2	0.32	2	0.52	0	0.00
Actinomucor elegans (Eidam) C.R. Benj. and Hesseltine (Z)	2	0.32	0	0.00	2	0.84
Gymnoascus sp. (A)	2	0.32	2	0.52	0	0.00
Humicola grisea Traaen (a.f.)	2	0.32	0	0.00	2	0.84
Cercospora sp. (a.f.)	1	0.16	1	0.26	0	0.00
Total	**623**		**386**		**237**	

* Common taxa in both beaches

N.I.F. – non identified fungi; (a.f.) – anamorphic fungi; (A) – Ascomycota; (Z) – Zygomycota; c.f.u. – colonies forming units; F.O.% – frequency of occurrence (in percentage)

Table 8.2. Taxa Richness, Shannon and Evenness indices for the soil mycota

	Richness (S)	Shannon (H′)	Evenness (E)
Forest pinewood soil	20	2.16	0.72
Vineyard soil	18	2.55	0.88

Table 8.3A. Frequency of occurrence of taxa detected in sand beaches

	Guincho and Rainha beaches		**Guincho beach**		**Rainha beach**	
Taxa	c.f.u	F.O.%	c.f.u.	F.O.%	c.f.u.	F.O.%
N.I.F (a.f.)	112	33.80	0	0.00	112	52.1
Penicillium spp. (a.f.) *	53	16.0	36	31.00	17	7.91
Mycelia sterilia *	43	13.0	28	24.10	15	6.98
Clasdosporium spp. (a.f.) *	24	7.30	16	13.80	8	3.72

Chalara sp. (a.f.) *	15	4.50	2	1.72	13	6.05
Acremonium spp. (a.f.) *	11	3.30	8	6.90	3	1.40
Trichoderma sp. (a.f.) *	11	3.30	7	6.03	4	1.86
Geotrichum spp. (a.f.) *	9	2.70	5	4.31	4	1.86
Alternaria sp. (a.f.) *	8	2.40	3	2.59	5	2.33
Beauveria sp. (a.f.)	7	2.10	0	0.00	7	3.26
Acladium sp. (a.f.)	4	1.20	0	0.00	4	1.86
Fusarium spp. (a.f.) *	4	1.20	3	2.59	1	0.47
Gliocladium sp. (a.f)	4	1.20	0	0.00	4	1.86
Scopulariopsis spp. (a.f.) *	4	1.20	3	2.59	1	0.47
Ulocladium sp. (a.f.) *	4	1.20	1	0.86	3	1.40
Chaeotobolisia sp. (a.f.)	3	0.90	0	0.00	3	1.40
Pestalotiopsis sp. (a.f.)	3	0.90	3	2.59	0	0.00
Phoma sp. (a.f.)	3	0.90	0	0.00	3	1.40
Absidia sp. (Z)	2	0.60	0	0.00	2	0.93
Aspergillus sp. (a.f.)	1	0.30	0	0.00	1	0.47
Mucor sp. (Z)	2	0.60	0	0.00	2	0.93
Gliomastix sp. (a.f.)	1	0.30	0	0.00	1	0.47
Pleurophragmium sp. (a.f.)	1	0.30	0	0.00	1	0.47
Scytalidium sp. (a.f.)	1	0.30	0	0.00	1	0.47
Botrytis sp. (a.f.)	1	0.30	1	0.86	0	0.00
Total	**331**		**116**		**215**	

* Common taxa in both beaches

N.I.F. – non identified fungi; (a.f.) – anamorphic fungi; (Z) – Zygomycota; c.f.u. – colonies forming units; F.O.% –frequency of occurrence (in percentage)

Table 8.3B. Frequency of occurrence of taxa detected in dry and wet sand from Rainha and Guincho beaches

	Guincho Beach				**Rainha Beach**			
	Dry Sand		**Wet Sand**		**Dry Sand**		**Wet Sand**	
Taxa	c.f.u.	F.O.%	c.f.u.	F.O.%	c.f.u.	F.O.%	c.f.u.	F.O.%
Absidia sp.(Z)	0	0.00	0	0.00	2	1.07	0	0.00
Acladium sp.(a.f.)	0	0.00	0	0.00	4	2.14	0	0.00
Acremonium spp. (a.f.) *	8	7.14	0	0.00	1	0.53	2	7.14
Alternaria sp.(a.f.) *	3	2.68	0	0.00	5	2.67	0	0.00
Aspergillus sp.(a.f.)	0	0.00	0	0.00	1	0.53	0	0.00
Beauveria sp. (a.f.)	0	0.00	0	0.00	3	1.60	4	14.29
Botrytis sp. (a.f.)	1	0.89	0	0.00	0	0.00	0	0.00

Cladosporium spp. (a.f.) *	15	13.39	1	25.00	5	2.67	3	10.71
Chaetobolisia sp. (a.f.)	0	0.00	0	0.00	0	0.00	3	10.71
Chalara sp. (a.f.) *	2	1.79	0	0.00	13	6.95	0	0.00
Fusarium spp. (a.f.) *	3	2.68	0	0.00	1	0.53	0	0.00
Geotrichum spp. (a.f.) *	5	4.46	0	0.00	3	1.60	1	3.57
Gliocladium sp. (a.f.)	0	0.00	0	0.00	4	2.14	0	0.00
Gliomastix sp. (a.f.)	0	0.00	0	0.00	1	0.53	0	0.00
Mucor sp. (Z)	0	0.00	0	0.00	2	1.07	0	0.00
Penicillium spp. (a.f.) *	36	32.14	0	0.00	14	7.49	3	10.71
Pestalotiopsis sp. (a.f.)	3	2.68	0	0.00	0	0.00	0	0.00
Phoma sp. (a.f)	0	0.00	0	0.00	3	1.60	0	0.00
Pleurophragmium sp. (a.f)	0	0.00	0	0.00	1	0.53	0	0.00
Scopulariopsis spp. (a.f.) *	3	2.68	0	0.00	0	0.00	1	3.57
Scytalidium sp. (a.f.)	0	0.00	0	0.00	0	0.00	1	3.57
Trichoderma sp. (a.f.) *	7	6.25	0	0.00	2	1.07	2	7.14
Ulocladium sp. (a.f.) *	1	0.89	0	0.00	3	1.60	0	0.00
N.I.F.	0	0.00	0	0.00	112	59.89	0	0.00
Mycelia sterilia *	25	22.32	3	75.00	7	3.74	8	28.57
Total	**112**		**4**		**187**		**28**	

* Common taxa in both beaches

N.I.F. non identified fungi; (a.f.) – anamorphic fungi; (Z) – Zygomycota; c.f.u. – colonies forming units; F.O.% - frequency of occurrence (in percentage)

Considering the selection media used 212 and 174 were the c.f.u. values obtained for the pinewood forest soil with RBA and with CZ, respectively. For the vineyard soil, the recorded c.f.u. values were 132 (RBA) and 105 (CZ).

Analysing the results obtained with MA and MYC, the c.f.u. values obtained for Guincho beach were 108 on MA and 8 on MYC. For Rainha beach, the corresponding c.f.u. values were 180 (MA) and 35 (MYC).

The taxa were classified as "very frequent" (> 20% of occurrence), "frequent" (10-20% of occurrence), and infrequent (<10% of occurrence) as used by Tan *et al.* (1989).

Soil fungi

The total number of fungal taxa registered in both soils was 26: 5 Zygomycota, 1 Ascomycota and 20 anamorphic fungi (Table 8.1). From these 26 taxa, 12 are common to both soils, all of them being anamorphic fungi (Table 8.1).

From the 26 taxa, 17 have been identified to species level. From the 8 *Aspergillus* spp., 4 have been identified: *Aspergillus niger* van Tieghem, *Aspergillus ochraceus* Wilhelm, *Aspergillus ustus* (Bain.) Thom and Church, *Aspergillus versicolor* (Vuill.) Tiraboschi. From the *Penicillium* spp., only *Penicillium frequentans* Westling has been identified.

Still concerning both soils, *Penicillium* spp. was very frequent (28.25%), *Aspergillus* spp. was frequent (15.09%) while there were 24 infrequent taxa.

In the pinewood forest soil, from the 20 taxa detected, 2 Zygomycota, 1 Ascomycota and 5 anamorphic fungi are exclusive to this soil. *Pencillium* spp. was very frequent (38.60%) and *Scopulariopsis* sp. (13.21%), *Trichoderma* sp. (10.62%), and *Aspergillus* spp. (9.84%) were frequent; the 16 other taxa were infrequent.The taxa isolated with RBA and CZ media were 19 and 12 respectively, 11 of them being common.

In the vineyard soil 18 taxa were detected, 6 of which are exclusive to this soil: 3 Zygomycota and 3 anamorphic fungi (Table 8.1). *Aspergillus* spp. was very frequent (23.63%), *Penicillium* spp. (11.39%) and *Rhizopus* sp. (10.13%) were frequent, and the 15 other taxa were infrequent (Table 8.1).The taxa isolated with RBA and CZ media were 18 and 14 respectively, all these 14 being common.

In general, all taxonomic groups of fungi are represented in soil. The most frequently isolated are members of the group anamorphic fungi, such as species of *Aspergillus, Geotrichum, Penicillium* and *Trichoderma* (Altas and Bartha, 1998). Also *Epicoccum, Fusarium*, *Gliomastix*, *Memnoniella* and *Stachybotrys* occur frequently as well as the Zygomycota *Absidia*, *Mortierella*, *Mucor*, *Rhizopus*, and *Zygorhynchus*. Common Ascomycota are *Chaetomium* and *Gymnoascus* (Carlile *et al.*, 2001).

In this study, *Penicillium* was more frequent than *Aspergillus* as would be expected for temperate areas (Kirk *et al*, 2001). Moreover all the taxa detected correspond to those already found in soils.

These results are also in accordance with the selected isolation method (serial dilutions) which generally favours the sporulating species (Davet and Rouxel, 2000).

In terms of the isolation media used, RBA was the preferable, as expected, because its source of sugar is a monosaccharide and the dye itself slows down the growth of the fungi. This effect limits the size of the colonies, thus making isolation easier.

The values obtained for taxa Richness were quite similar: 20 for the pinewood forest soil and 18 for the vineyard soil (Table 8.2).

The vineyard soil presented higher values for Shannon and Evenness indices (**H'**=2.55 and **E**=0.88), as compared to the pinewood forest soil (**H'**=2.16 and **E**= 0.72), suggesting higher fungal diversity in the vineyard soil (Table 8.2).

Again considering these two soils, Sorenson similarity Index for the mycota was 0.63 (**j**=12, **a**= 20 and **b**=18), indicating a mean value of similarity between the mycota of these soils.

Analysing the results achieved with the Cholodny-Rossi method, only 10 out of the 36 slides buried in the pinewood soil forest were observed, the remaining having been lost. The microbial colonization observed on the slides of the laboratory assays was similar to the "in situ" assays for the same soil. However the structures from the laboratory assays were more preserved.

Under the microscope, soil particles, bacteria, actinomycetes, fungi spores and mycelia with and without septa and also with clamp connections, as well as chlamydospores were observed (Figs. 8.1 to 8.5). Clamp connections, characteristic of Basidiomycota were observed only on the slides from pinewood forest soil.

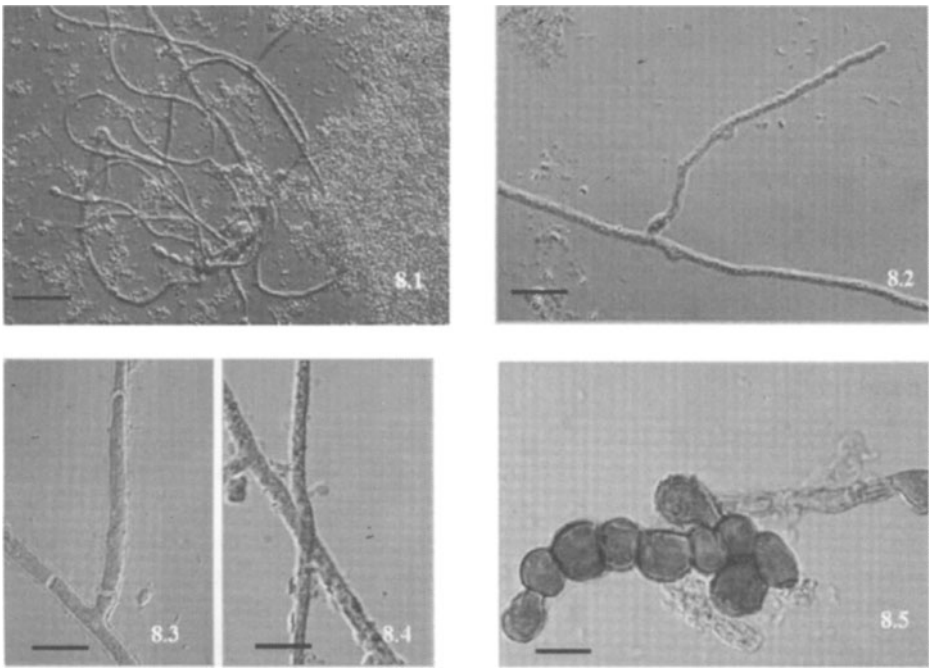

Fig. 8.1. Bacteria and network of hyphae from actinomycetes (bar-12.50 μm), **8.2.** Bacteria and septate hyphae of fungi with clamp connections (bar-12.50 μm) **8.3.** Septate hyaline hyphae of fungi (bar-7.80 μm), **8.4.** Non septate hyaline hyphae of fungi (bar-16.60 μm), **8.5.** Chain of chlamydospores of fungi (bar-7.50 μm). Slides of samples from Pinewood soil forest (8.2 and 8.3); and from Vineyard soil (8.1, 8.4, and 8.5) (Azevedo, 2003)

Mycelia of Basidiomycota are most frequent under trees (Kirk *et al.*, 2001) and are usually hard to identify since the isolates seldom sporulate in culture (Carlile *et al.*, 2001). They often account for more than half of fungal biomass

and activity in a soil, but are usually not reported in soil fungi inventories. Their diversity is much better assessed in the context of inventories of mycorrhizal and wood/litter-decomposing fungi or, more recently, by molecular approaches (Gams, 2007).

Our results are in accordance with Kirk *et al.* (2001) and suggest the existence of symbiotic association between fungi mycelia and roots of pine trees (ectomycorrhizas).

On the slides from the vineyard soil a dense network of actinomycetes and fungi mycelia without septa was observed, suggesting typical soma of some Zygomycota.

Actinomycetes grow, preferentially, on basic or neutral soils (Altas and Bartha, 1998). Accordingly, the vineyard soil showed pH 6, close to the optimal pH for the growth of actinomycetes.

Relative to the orientation of soil microorganisms on the slides, it was observed that the microbial colonization of the soil by bacteria, actinomycetes and fungi is detected mainly on the medium part or near the top of the buried zone of the slides and occurs either in association with soil particles or in juxtaposition to each other.

Atlas and Bartha (1998) consider that a clean glass slide surface is not selective and acts like a surface of mineral particles of soil, therefore the types and proportions of organisms that adhere to the slide can be considered representative of the community in general. It can thus be assumed that the microbial organisms found in this study are representative of the communities actually existing in the soils under analysis.

Sand fungi

As can be seen in Table 8.3 A, 13 taxa were detected at Guincho beach and 23 at Rainha Beach; 11 out of the total 25 taxa detected, are common to the sands of both beaches.

Very frequent fungi at Guincho beach were *Penicillium* spp. (31.00%). *Mycelia sterilia* (24.10%) and *Cladosporium* spp. were frequent (13.80%), while 10 taxa were infrequent.

At Rainha beach, non identified fungi (N.I.F.), classified as anamorphic fungi (a.f.) were very frequent (52.10%); the remaining 21 taxa and *Mycelia sterilia* were infrequent (Table 8.3 A).

Two anamorphic fungi are exclusive of Guincho beach. Two Zygomycota, 9 anamorphic fungi as well as all the N.I.F (a.f.) are exclusive of Rainha beach (Table 8.3 A).

Considering the selection media used, from Guincho beach 13 taxa were isolated on MA and 4 on MYC, and for Rainha beach 20 taxa were isolated on MA and 7 on MYC. Four taxa were common in both media, in each situation (Guincho and Rainha beaches).

Comparing the results obtained with these two media, a smaller number of c.f.u. was obtained with MYC, compared with MA. As expected, MYC supplemented with cycloheximide inhibited the growth of the fungi that do not tolerate the used concentration of this fungicide, only allowing the growth of resistant fungi.

Richness, Shannon and Evenness diversity indices are listed on Table 8.4. The taxa Richness was always higher for Rainha beach (S_{RT}=23) as well as for the dry sand of both beaches (S_{RD}=20), (S_{GD}=13). The Shannon indices for Guincho environments were very discrepant, being high for the dry sand (H'_{GD} = 2.01) and particularly low for the wet sand (H'_{GW}= 0.57). This result may be due to the characteristics of Guincho beach described in Experimental section (exposed to winds and high waves).

Table 8.4. Taxa Richness, Shannon and Evenness indices for the mycota from the sands of Guincho and Rainha beaches

Beach	Sands	Richness (S)	Shannon (H')	Evenness (E)
G	D	13	2.01	0.78
	W	2	0.57	0.82
	T	13	2.11	0.82
R	D	20	1.72	0.57
	W	10	2.09	0.91
	T	23	2.15	0.69

G – Guincho; R – Rainha ; D – Dry; W – Wet; T – Total

The values for Evenness were 0.82 e 0.69 for Guincho and Rainha respectively. These results indicate that the species were more evenly abundant in Guincho than in Rainha.

Considering the two types of sand the values for Evenness were similar for Guincho (E_{GD}= 0.78 and E_{GW}=0.82) and particularly different for Rainha (E_{RD}=0.57 and E_{RW}=0.91). These results indicate that the species were evenly abundant in both sands of Guincho and in wet sand of Rainha as compared to dry sand.

Concerning the mycota similarity between the two beaches, Sorenson index was 0.61 (**j**= 11, **a**=13 and b=23), indicating a mean value of similarity.

Comparing the two types of sand, Sorenson similarity index showed a low value for the mycota of Guincho beach and a mean value for Rainha beach. These values were 0.27 (j=2, a=13 and b=2) and 0.48 (j=7, a=20 and b=9) respectively.

Comparing the results of our survey with studies from sandy beaches in Spain referred in introduction (Izquierdo *et al.*, 1986 cited by WHO, 2003), we may say that the potentially pathogenic genera of *Penicillium*, *Aspergillus* and *Cladosporium* were also isolated from sandy beaches in Portugal. With respect to the survey of Roses Codinachs *et al.* (1988), in which the most frequently

isolated genera were *Penicillium*, *Aspergillus*, *Cladosporium Alternaria*, *Mucor*, *Monilia*, *Cephalosporium*, *Verticillium* and *Chrysosporium*, with exception of the last 4 genera referred above, all the others were also isolated in this study, being *Penicillium* classified in our work as frequent, and *Cladosporium*, *Alternaria* and *Mucor* as infrequent taxa (Table 8.3 A). In the survey of Larrondo and Calvo (1989) the most commonly isolated genera were *Penicillium*, *Cladosporium*, *Aspergillus*, *Acremonium*, *Alternaria* and *Fusarium*; these genera were also identified in our study but were all classified infrequent, except *Penicillium* (frequent).

This study included one method for the isolation of dermatophytes, a group of interest in environments frequented by humans, such as sandy beaches. Few keratinophlilic fungi were isolated from soils (*Scopulariopsis*) and sands (*Scopulariopsis* and *Scytalidium*), none of them being dermatophytes. However, these results could be qualitatively and/or quantitatively influenced by the period used to collect the samples (wintertime). In summertime beaches become more populated by animals and people, thus being more probable the isolation of dermatophytes due to an increase of keratinous debris (São José *et al.*, 1994).

Conclusions and future perspectives

This study, based on classic isolation and taxonomic approaches, provided only a partial knowledge of soil and sand mycota since only the culturing and sporulating fungi isolates could be identified through the use of illustrated keys.

For both soils and sands the very frequent fungi detected belong to the group of anamorphic fungi as can be seen in the following table:

Common mycota	Soil exclusive mycota	Sand exclusive mycota
Absidia (Z)	*Actinomucor* (Z)	*Acladium* (a.f.)
Mucor (Z)	*Mortierella* (Z)	*Beauveria* (a.f.)
Acremonium (a.f.)	*Rhizopus* (Z)	*Chaeotobolisia* (a.f.)
Aspergillus (a.f.)	*Cercospora* (a.f.)	*Chalara* (a.f.)
Alternaria (a.f.)	*Dorotomyces* (a.f.)	*Gliomastix* (a.f.)
Botrytis (a.f.)	*Humicola* (a.f.)	*Pleurophragmium* (a.f.)
Cladosporium (a.f.)	*Memmoniella* (a.f.)	*Scytalidium* (a.f.)
Fusarium (a.f.)	*Myrothecium* (a.f.)	*Ulocladium* (a.f.)
Gliocladium (a.f.)	*Stachybotrys* (a.f.)	
Penicillium (a.f.)	*Torulomyces* (a.f.)	
Pestalotiopsis (a.f.)	*Verticillium* (a.f.)	
Phoma (a.f.)	*Gymnoascus* (A)	
Scopulariopsis (a.f.)		
Trichoderma (a.f.)		

(Z) Zygomycota (a.f.) anamorphic fungi (A) Ascomycota

In this study 34 taxa were detected (5 Zygomycota, 1 Ascomycota and 28 anamorphic fungi). The common mycota to soils and sands consist of 14 taxa (2 Zygomycota and 12 anamorphic fungi).

Three Zygomycota, 8 anamorphic fungi and 1 Ascomycota are exclusive of the soil while 8 anamorphic fungi are exclusive of the sand.

The Sorenson index for the soil and sand mycota was 0.55 (**j**= 14, **a**= 26 and **b**= 25), indicating a mean value of similarity.

In respect to the sand mycota, three facts can be stressed: the dry sand provided a higher number of isolations as compared to the wet sand (Table 8.4); the absence of dermatophytes in both beach sands; the isolation of keratinophylic fungi, like *Scytalidium* and *Scopulariopsis*, this one also in soils (Tables 8.1 and 8.3 A).

Most of the isolated soil and sand fungi are saprobes, and some of them may be potentially pathogenic to animals and humans like, for example: *Absidia, Mucor, Acremonium, Aspergillus, Cladosporium, Curvularia, Geotrichum, Penicillium, Scopulariopsis* and *Scytalidium.* On the other hand, *Alternaria, Botrytis, Fusarium, Pestalotiopsis, Phoma* and *Verticillium* are among the most cited phytopathogens (Azevedo, 2003; Figueira, 2004).

Some isolates referred in this study belong to genera from which producers of a large variety of compounds are known. These compounds can be antibiotics (*Acremonium* and *Penicillium*), enzymes (*Aspergillus, Rhizopus* and *Trichoderma*), organic acids (*Aspergillus, Fusarium* and *Rhizopus*), as well as some important mycotoxins (*Aspergillus, Alternaria, Cladosporium, Fusarium, Trichoderma* and *Stachybotrys*) (Kendrick, 2000).

Still others are reported to be important agents of biocontrol, like species of the genera *Alternaria* (Daigle and Coninck, 2002), *Beauveria* (Kirk *et al.*, 2001), *Fusarium* (Carlile *et al.*, 2001; Daigle and Connick, 2002), *Trichoderma* (Papavizas, 1985; Chet *et al.*, 1987, cited by Samuels, 1996) and *Verticillium* (Kendrick, 2000). *Gliocladium roseum* has the potential to be used as a biocontrol agent of the phytopathogen *Botrytis cinerea* (Monaco *et al.*, 1999).

Hence, in the future, biochemical studies should be performed with some of the isolates obtained within the scope of this study, in the search for their potential in the production of biotechnological compounds.

As a large number of anamorphic fungi were detected in this survey, a complement to this study would be achieved by the application of molecular approaches, although they also have limitations. These are due to the shortage of authenticated reference sequences, as well as to uncertainties about the taxonomy of many fungi groups. It is therefore important to bear in mind, as referred by Brigde and Spooner (2001) that the lifestyle of the organism under study is an important point to be considered in the selection and interpretation of molecular approaches.

Soil fungi have been studied in the last years by several authors, namely by Kullnig *et al.* (2000), Viaud *et al.* (2000), and Anderson and Parkin (2007). Although presenting different approaches, their papers have in common the use of the internal transcribed spacers (ITS) of ribosomal genes. As a large number of species are yet to be found, and knowing that the analysis of their rRNAs is mandatory for the continuing reassessment of genera, the future application of identical approaches to the fungal diversity found in this study will, hopefully, be our contribution for this goal.

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CHAPTER - 9

ROLE OF MYCORRHIZAL FUNGI IN GROWTH PROMOTION OF CROP PLANTS

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Introduction

Living organisms in the biosphere exhibit a number of interactions, which either alter their environment and/or the size, and composition of each other's populations. Of these, perhaps the most striking relationship is 'symbiosis' in which the partners live in a state of physical and physiological equilibrium and derive benefit from each other. One such symbiotic association is the mycorrhiza.

Mycorrhiza is the symbiotic association between the fungi and the roots of the higher plants (Frank, 1885). This relationship benefits the plant growth by enabling a greater proportion of available nutrients in the soil to be absorbed into the plant. The fungal partner gets photosynthetic sugars as food from the plant which in turn acquires an array of benefits ranging from better uptake of phosphorus and relatively immobile micronutrients like zinc and copper (Norris *et al.*, 1991, 1992), increase in nitrogen fixing capacity of leguminous plant species, salinity and drought tolerance, maintenance of water balance, increased rate of photosynthesis to overall increase in plant growth and development. The beneficial role of AM association in phosphorus uptake and growth response under phosphorus limiting conditions has been well established for agricultural crops (Frey and Schüepp, 1992; Jakobson *et al.*, 1994). Endomycorrhiza can modify the root architecture to give a root system, which is better adopted for uptake of mineral nutrient and water (Berta *et al.*, 1990), as well as increasing hormone production.

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Mycorrhizae increases soil CO_2 level and contribute substantial amount of the total stand respirator. Glomalin produced by the hyphae of AMF plays a major role in maintaining stability and aggregation of soil (Wright and Upadhyaya, 1996, 1998).

The bi-directional transport leads to an enhanced plant growth and the completion of the fungal life cycle. Mycorrhiza protects the plants from higher tolerance of high soil temperatures and root-borne pathogens. In eutrophic soil these plants can take up nitrogen in the form of ammonia. Seedlings, which are colonized by AM fungi, perform better during transplantation. The mycorrhizal plants are also more tolerant towards heavy metal toxicity. For these reasons, mycorrhizal symbiosis is attractive systems in agriculture, flori- and horticulture, arboriculture, viticulture and forest management to enhance crop and wood production in the sense of a 'sustainable agriculture' and restoring 'soil fertility'. These fungi are potential 'biofertilizers' and 'bioprotectors' to enhance plant growth, yield, as soil conditioners (soil fertility) and play vital role in sustainability of the fast degrading environment (Ravolanirina *et al.*, 1989; Bagyaraj and Varma, 1995).

A large number of beneficial endophytes are likely to exist in nature. The best available example to date is possibly *Piriformospora indica* (Shende *et al.*, 2006). *P. indica,* a new growth promoting endophyte was discovered by Varma *et al.* (1998) from the rhizospheric soil of two xerophytic plants, viz., *Prosopis julifera* and *Zizyphus nummularia* of Rajasthan, which resembles AMF in many respects and used as a potential bioprotector and biofertilizer for the plants. The genus belongs to Basidiomycota. During the screening of glomaceous fungi for their culture on agar medium, this fungus grew with scanty mycelium and latter formed typically pear-shaped spores, and thus, named as *P. indica* (Verma *et al.*, 1998). It is the only growth promoting fungus, which can be grown on the axenic media. For this reason, it is an important tool for the better understanding of mycorrhizal symbiosis and molecular mechanism. Rai *et al.* (2001) reported the positive growth response of the *Spilanthus calva* and *Withania somnifera* by inoculation of *Piriformospora indica* in a field trial. Rai *et al.* (2004) also reported the antifungal potential of *Spilanthus calva* after inoculation of *P. indica*. The growth promotion of *Adhatoda vasica* Nees by *P. indica* is evaluated by Rai and Varma (2005).

Being a potential plant growth promoter, the fungus has attracted the researchers, all over the world to harness its fullest potential in the field of sustainable agriculture. A review of literature vouches that a significant contribution has been made on various aspects of *P. indica* with particular reference to its growth promoting potential (Sahay *et al.*, 1998; Sahay, 1999; Varma *et al.*, 1999a; Sahay and Varma, 2000; Rai *et al.*, 2001; Rai and Varma, 2002; Singh *et al.*, 2002; 2003a). The plant hormones like auxins and cytokinins also play a significant role in mutualistic interactions between symbionts, in case of *P. indica* (Vadassery *et al.*, 2008.). It is also possible that *P. indica* promotes

growth and involved in nitrogen accumulation by expressing enzyme nitrate reductase and starch degrading enzyme glucan water dikinase (Sherameti *et al.*, 2005). A leucine rich repeat protein is required for growth promotion and enhanced seed production mediated by endophytic fungus *P.indica* (Shahollari *et al.*, 2007). Apart from growth promotion, *P. indica* is also involved in localised and systemic induction of host resistance (Waller *et al.*, 2005).

Blechert *et al.* (1999) gave the first remarks on the symbiotic interaction between *P. indica* and terrestrial orchids. Since then a remarkable progress has been made to utilise biotechnological potential of this culturable fungus in the field of horticulture and agriculture. As a matter of fact, the fungus *P. indica* has tremendous potential in the field of industries dealing with tissue culture and also in the field of agriculture.

A lot of work done in the past few decades has enabled these fungi to emerge as a potential biofertilizer, a cheap and environment-friendly alternative to petroleum based chemical fertilizers. This aspect especially gains significance for a developing country like India where judicious and large scale utilization of this technology can prove very useful for getting maximum and long-term gains in various wasteland reclamation, reforestation and afforestation programmes apart from giving a much needed thrust in the production of important agricultural crops on which the economy of the country is dependent.

Abundance and distribution

According to the molecular data, AMF originated in devonian time (Simon *et al.*, 1993) and there is a convincing fossil record of earlier plants (Berch, 1986; Stubblefield *et al.*, 1987; Haas *et al.*, 1994) which provide evidence that AMF were present in early land plants (Remy *et al.*, 1994).

Mycorrhizal associations are ubiquitous in nature and are known to colonize roots of plants belonging to more than 90 percent of plant families and found in a broad range of habitats. These includes ecosystem, ranging from aquatic (Bagyaraj *et al.*, 1979; Khan and Belik, 1995), to deserts (Williams and Aldon, 1976; Singh and Varma, 1980; Neeraj *et al.*, 1991), from low land tropical rain forests (Hogberg, 1982; Janos, 1987) to high altitudes (Christie and Nicolson, 1983; Laursen, 1985) and in the canopy epiphytes and also in a wide spectrum of temperate and tropical plant species. They occur more frequently in cultivated soils usually in top 15-30 cm (Redhead, 1977; Bagyaraj *et al.*, 1979). They are of common occurrence in the plant kingdom and are reported from bryophytes, pteridophytes, many gymnosperms and most angiosperms (Harley, 1991; Cazres and Trappe, 1993). They are absent only from a few plant families, *viz.*, *Aizoceae*, *Amaranthaceae*, *Cruciferae, Fumariaceae*, *Pinaceae*, *Portulaceae*, *Resitonaceae*, *Urticaceae* and *Zygophyllaceae* (Tester *et al.*, 1987; Koide and Schreiner, 1992).

Many edaphic factors such as soils type (Frey and Ellis, 1997), soil fertility (Bolgiano *et al.*, 1983), and pH (Clark, 1997), affect the extent of colonization.

Soil disturbance due to the tillage can adversely influence the distribution of AMF colonization (Anderson *et al.*, 1987; McGonigle *et al.*, 1990; Kabir *et al.*, 1999). Similarly, pesticide/ insecticide application is reported to alter fungal diversity significantly (Johnson *et al.*, 1992). Abundance and worldwide distribution of AM fungi is an indication of its enormous capacity to survive.

Symbiosis establishment

For establishment of AM-plant symbiosis, probably molecular signalling events must occur that lead to various physiological and anatomical changes in both symbionts. The molecular communication may be exchanged between the fungus and host plant in the rhizosphere, the rhizoplane, the root epidermis and cortex (Azcon and Ocampo, 1981; Smith and Gianinazzi-Pearson, 1988; Siqueira *et al.*, 1991). Signals diffuse from host plants affect spore germination, germ tube extension and its direction in the rhizosphere. Signals on the rhizoplane influence the adhesion and penetrations and signals inside the root may control the spread and intensity of infection, formation of arbuscules, vesicles and spore and the rate of carbohydrate transfer to the fungus.

An early event in the recognition of host plants by AM fungi is a chemotropic differential hyphal morphogenesis elicitated by the roots exudate of host plants prior to appresorium formation (Giovannetti *et al.*, 1993b).

The establishment of colonization is preceded by some growth of the fungi on the root surface of the host plant. Infection occurs mainly by direct penetration of the outermost host cell-wall with the help of cellulolytic and pectinolytic enzymes of fungal origin or root hairs through which some hyphae may enter *via* the intercellular spaces and seem to be influenced by the features of the host cell surface (Bonfante, 1988). After the fungal penetration of the cell-wall, the host plasmalemma elongates and extends around the invading fungus. With cell to cell contact between the two symbionts the external hyphae progress towards the root cortical parenchyma, where they proliferate intracellularly by destroying the middle-lamella (Gianinazzi-Pearson *et al.*, 1981) and usually swells at the point of contact to form more or less well defined appressorium, 20-40 μm long from which the infectious hyphae develop and penetrate the cell-wall of the plant cell (Garriock *et al.*, 1989). Arbuscules are branched haustoria-like structure formed early in the association resulting from repeated dichotomous branching of fungal branches. The outer layers of plant roots vary greatly in the amount of pectin and cellulose in their walls, which seems to play a vital role in the microbial entry into the plant systems (Jarvis *et al.*, 1988).

Other structures produced by AM fungi include vesicles, auxiliary cells, extrametrical hyphae and spores. Vesicles are thin-walled, lipid filled structures that usually form in intercellular spaces. Their primary function is thought to be for storage; however, vesicles can also serve as reproductive propagules for the fungus.

The AM fungi may produce an extensive network of extrametrical hyphae (Sylvia, 1990) and can significantly increase phosphorus-inflow rates of the plants they colonize. In many ecosystems these form connecting channels between plants situated quite distantly and help in mineral flow. These hyphae due to their smaller diameter can reach areas where root hairs cannot. One of the highest hyphal estimates in soil is 111 m/cm^3 for a prairie community, for which a hyphal dry-weight of less than 0.5 mg/gm is recorded (Miller *et al.*, 1995); thus, giving the mycorrhizal plants an edge in nutrient uptake over the non-mycorrhizal ones.

Since these fungi lack sexual reproduction recognition, characterization and classification is based upon their chlamydospores which show variation in size, shape, colour and wall characteristics depending upon the species (Berch, 1986; Rani and Mukerji, 1988; Morton and Benny, 1990; Mukerji and Kapoor, 1990; Morton *et al.*, 1992; Morton, 1993; Morton and Bentivenga, 1994; Mukerji, 1996).

Plant-host specificity

The AMF are not host-specific. Nonetheless, there is mounting evidence that "host preference" is an important characteristic of AM symbiosis (Dhillion, 1992). Therefore, it is important to distinguish among specificity (innate ability to colonize), infectiveness (amount of colonization) and effectiveness (plant response to colonization) of plant fungus symbiosis. Host preference may be under the genetic control of the host, the fungus, or most likely a complex interactive effect of both symbiotic partners with soil edaphic factors. Bever *et al.* (1996) reported host-dependent sporulation among common lawn plants. Host genotype variation in root colonization and plant response also has been demonstrated for wheat (Hetrick *et al.*, 1993), pea (Martensson and Rydberg, 1995) and citrus (Graham and Eissenstat, 1998).

Mycorrhizal dependence of plants

AMF fungi form mycorrhizal association with majority of the plant species, except, members of Cruciferae, Cyperaceae and Chenopodiaceae (Mosse, 1981; Mukerji *et al.*, 1982; Kapoor *et al.*, 1988; Rani and Mukerji, 1990; Ragupathy and Mahadevan, 1993). Members of Amaranthaceae known earlier as non-mycorrhizal (Gerdemann, 1968) were found to be mycorrhizal in the tropical plains of Tamil Nadu, India (Ragupathy and Mahadevan, 1993).

A large difference exists between plant species in their dependence on AM fungi for phosphorus uptake and growth. Plant species having thick, fleshy roots with few root hairs such as Cassava (*Manihot esculenta*), Citrus (*Citrus* species), Sweetgum (*Liquidambar styraciflua*), Grape (*Vitis vinifera*) and most legumes are considered as mycorrhiza dependent (Mosse, 1981).

Yost and Fox (1979) reported greater dependence on AMF fungi for phosphorus uptake by *Manihot esculenta* and *Stylosanthes* species compared

with cowpea (*Vigna unguiculata*), onion (*Allium cepa*), soyabean (*Glycine max*) and *Leucaena* species. According to Bowen (1980), all economically important tropical crops are mycorrhizal with the exception of flooded rice (*Oryza sativa*). Under conditions of very low levels of available phosphorus, the grasses may be as mycorrhiza dependent as the legumes or more woody crop species such as cassava. According to Lin Xian-Gui and Hao Wen- Yin (1988), grape (*Vitis vinifera*) has high mycorrhizal dependency.

Most of the plants show variation in their dependency to the mycorrhizal infection. *Ananas comosus, Cassia reticulata, Chloris gayana, Glycine max, Lycopersicum esculentum, Sesbania pachycarpa, S. sesban, Sorghum bicolor* and *Zea mays* are marginally dependent to the arbuscular mycorrhizal fungi, whereas *Acacia mangium, Colocasia esculenta, Gliricidia sepium, Leucaena retusa* and *Sesbania grandiflora* are moderately dependent. *Albizia feruginea, Allium cepa, Azadirachta indica, Cajanus cajan, Carica papaya, Cassia siamea, Coffea arabica, Enterolobium cyclocarpum, Leucaena diversifolia, Leucaena trichodes, Paraserianthes falcataria, Senna spectabilis* and *Sauropus androgynus* are highly dependent and *Leucaena leucocephala, Manihot esculenta* and *Sophora chrysophylla* are very highly dependent to the arbuscular mycorrhizal fungi (Miyasaka and Habte, 2001).

Finally, arbuscular mycorrhizal fungal technology is the need of the hour in order to achieve the targeted output of food grains, pulses, oil seeds and other economically important plants. A few attempts have demonstrated the association of AM fungi with medicinal plants but the effects of AMF on the medicinal values received little attention. The medicinal plants are of immense importance as a number of drugs are manufactured from them, which have direct concern with human health.

Mycorrhization in micropropagation

Micropropagation is an excellent tool for the production of homogeneous, genetically improved orchard, endangered medicinal plants and ornamentai crops. The rooting of *in vitro* produced shoots is often the limiting step during micropropagation.

Roots of plants produced by micropropagation are devoid of mycorrhizal fungi. The application of AM inoculum to cultures could therefore produce modifications in root morphology and dynamics, which would aid the establishment and growth of plantlets. The acclimatization phase raises problems concerning survival and development of the plantlets. The survival rate and initial 'transplant shock' on transfer of the plants to field is very high. Often stunted growth leads to non-recovery of the plants and is attacked by soil fungi. Today about 50 per cent flori-horticulture plants are produced by micropropagation techniques, but at weaning stage about 10-40% of plantlets either die or do not attain market standards, causing significant losses at the commercial level. In the vegetative propagation of many crops, rooting of (micro) cutting is the

crucial step (Klerk and Brugge, 1992). Such problems can be overcome by the application of AMF to the micropropagated plantlets. The use of micropropagation for production of elite stock of strawberry has opened up more possibilities (Vestberg, 1992).

Varma and Schüepp (1995) and Lovato *et al.* (1995) stated that inoculation of micropropagated plantlets with active culture of AMF appears to be critical for their survival and growth. This avoids '*Transient transplant shock*' and shunted growth on transfer to the field (Lovato *et al.*, 1995).

Inoculation of AMF to nursery plants has been proven to both necessary and feasible, and it has been extended to micropropagated plants (Kierman *et al.*, 1984; Varma and Schüepp, 1995). The technique of mycorrhization has been applied recently for the growth promotion of woody plants (Salamanca *et al.*, 1992). Habte *et al.* (2001) studied the role of AMF in early forest tree establishment. Gianinazzi *et al.* (1990) reported the beneficial effect of *G. mosseae* and *G. intraradices* to the seeds of *Ampelopsis ash*, *Berberis*, *Chamaecyparis*, *Lilac liquidambar* and *Hick yew*. Mycorrhizal inoculants have already been commercialised (Sieverding, 1991).

There are several reports that inoculation with mycorrhizal inoculum to micropropagated shrub legumes shortened their acclimatization process, and their shorter cycle of high value (Atkinson *et al.*, 1994). Usoukainen and Vestberg (1994) found that mycorrhized apple plants were more uniform in size, a fact also found by several other working with fruit trees (Vestberg and Estaun, 1994). From industrial and economic point of view the uniformity of the plants is a desirable characteristic in any nursery, allowing for a homogeneous classification of the stock. Lin *et al.* (1987) based on a large scale survey on micropropagated potato, strawberry, blackberry, apple, rose, ginger, pine, apple, with AMF strains found blackberry and apple to be the most promising plants, from which it would be feasible and advantageous to produce mycorrhizal nursery stocks.

Micropropagated plants inoculated in *ex vitro* experiments are highly mycorrhiza dependent. This fact has been documented in high-value plants like grape vines (Ravolanirina *et al.*, 1989; Schubert *et al.*, 1990; Schllenbaum *et al.*, 1991), oil palm (Blal *et al.*, 1990), apple (Branzati *et al.*, 1992; Usoukainen and Vestberg, 1994), plum (Fortuna *et al.*, 1992), pineapple (Lovato *et al.*, 1992; Usoukainen and Vestberg, 1994), avacado (Azcon-Aguilar *et al.*, 1992), strawberry (Varma and Schüepp, 1994b), raspberry (Varma and Schüepp, 1994b), hortensia (Varma and Schüepp, 1994a; Varma and Schüepp, 1995), woody legumes (Salamanca *et al.*, 1992), rhododendron (Lemoine *et al.*, 1992). Plants inoculated with AM spores increase the survival rate and growth in potted condition. *Vitis vinifera* plantlets inoculated with *Glomus epigeous* significantly increased percentage infection, biomass, photosynthesis of host and increased resistance to downy mildew disease, compared with control. Therefore, there is high potential for introducing AMF into the micropropagation system of these plants and of other high-value plants.

A survey of current literature reveals that inoculation of arbuscular mycorrhizal fungi into the roots of micropropagated plantlets play a beneficial role (Blal *et al.*, 1990; Schubert *et al.*, 1990; Azcon-Aguilar *et al.*, 1994; Declereck *et al.*, 1994; Varma and Schüepp, 1994a,b; Gribaudo *et al.*, 1996; Martin *et al.*, 1996; Vestberg and Uosukaninen, 1996; Budi *et al.*, 1998; Naqui and Mukerji, 1998; Gange and Ayres, 1999; Vosatka *et al.*, 1999 Table 9.1).

Table 9.1. Arbuscular mycorrhizal fungi used as inoculant for various micropropagated plantlets (Rai, 2001).

Author	Year	Mycorrhizal species	Host plant
Schubert *et al.*	1987	*G. caledonium* *G. clarum, G. versiforme*	*Vitis berlandieri X* *V. vinifera* (Kober 5BB)
Ravolanirina *et al.*	1989	*Gigaspora margarita* (LPA 2) *Glomus mosseae (LPA 5)* *G. caledonium* (LPA 12) *G. fasciculatum* (LPA 7)	Vine *(Vitis vinifera)* and *oil-palm* *(Elais guinnensis)*
Schubert *et al.*	1990	*G. fasciculatum, G. caledonium* *G. monosporum, Glomus sp.* E3, *G. constrictum, G. occultum* and *G. vesiforme*	Kiwi fruit *(Actinidia deliciosa)* Grapevines
Lovato *et al.*	1992	*Glomus sp* (LPA 21), *G. intraradices* (LPA 8)	*Grapevines rootstocks* Pine apple
Arines and Ballester	1992	*G. aggregatum* and *G. deserticola*	*Prunus avium, Spiraea vulgaris, Syringa japonica*
Azcon-Aguilar *et al.*	1992	*Glomus fasciculatum,* *G. deserticola*	Avocado *(Persea americana)*
Bouhired *et al.*	1992	*Glomus fasciculatum* *G. intraradices* (LPA 8) *G. isolate* (LPA 21)	Date palm *(Phoenix dactylifera)*
Branzanti *et al.*	1992	*G. fasciculatum* *G. mosseae, G. intraradices*	Apple (M 9, M 26, golden)
Fortuna *et al.*	1992	*G. mosseae, G. coronatum* *G. caledonium, G. sp.* strain A6	Plum rootstock *(Prunus cerasifera)*
Guillemin *et al.*	1992	*G. sp.* (LPA 21), *G.* sp. (LPA 22) *G. clarum* (LPA 16) *G. sp.* (LPA 25), *Scutellospora pellucida* (LPA 20)	Pineapple (3 varieties)
Jaizme-Vega	1992	*G. mosseae*	Banana *(Musa acuminata)*
Honrubia and Morte	1992	*Glomus fasciculatum*	*Tetraclinis articulata*
Sbrana *et al.*	1992	*Glomus sp.* strain A6	Apple and Peach rootstocks

Schubert *et al.*	1992	*Glomus* sp. *strain E3*	Kiwi fruit *(Actinidia deliciosa)*
Tisserant and Gianinazzi-Pearson	1992	*Glomus fasciculatum*	*Platanus acerifolia*
Williams *et al.*	1992	*G. intraradices* Finn 98 *Glomus* sp. Finn 128 *G. geosporum*	Strawberry
Vestberg	1992	*G. intraradices* and *G. mosseae*	Strawberry
Declerck *et al*	1994	*Glomus versiforme*, *G. intraradices*	Banana and sugarcane
Varma & Schüepp	1994b	*Glomus sp.*	*Hortensia sp.*
Azcon-Aguilar *et al.*	1996	*G. deserticola*	*Annona cherimola*
Morte *et al.*	1996	*Glomus fasciculatum*	*Tetraclinis articulata*
Rapparini *et al.*	1996	*Glomus sp.*	*Pyrus communis*
Vestberg and Uosukainen	1996	*G. mosseae* (V 57 (BEG 29) *G. hoi* V 98 (BEG 48), *G. caledonium* V 126b *G. fistulosum* V 128 (BEG 31)	Greenhouse rose 'Mercedes'
Cordier *et al.*	1996	*G. intraradices* and *G. caledonium*	*Prunus avium*
Dolcet-Sanjuan *et al.*	1996	*G. mosseae* and *G. intraradices*	Walnut *(Juglans regia)*
Uosukainen and Vestberg	1996	*G. claroideum* *G. fistulosum*	Crab apple cv Marjatta
Rancillac *et al.*	1996	*Gigaspora rosea* *G. mosseae*	*Allium cepa*
Gribaudo *et al.*	1996	*G. mosseae*	Grapevine (Kober 5 BB)
Mark *et al.*	1999	*G. fasciculatum, G. macrocarpum* *Glomus mosseae*	*Casuarina equisetifolia*
Rai and Varma	2002	*G. mosseae, G. caledonium* *G. intraradices*	*Withania somnifera*
Marin *et al.*	2003	*G. mosseae, G. intraradises*	*Diospyros kaki*
Quatrini *et al.*	2003	*G. mosseae*	*Citrus limon*
Lovato *et al.*	2006	*G. deserticola G. intraradices*	*Prunus avium*
Binet *et al.*	2007	*G. mosseae*	*Olea europaea*
Da Silva *et al.*	2008	*Scutellospora heterogama* SCT120E, *Gigaspora decipiens SCT 304*A, *Acaulospora koskei SCT400*A, *Enterophospora colombiana SCT*115	*Zingiber officinale*

Beneficial effects of AM

Role in phosphorus uptake

Phosphorus is one of the most important macronutrient for plant life. It occurs as a part of nucleus, DNA and RNA. As part of phospholipid it constitutes plant membranes. It is part of high energy molecules like ADP, ATP, NADP and NAD which in turn govern all the oxidation-reduction reactions like photosynthesis, respiration, nitrogen metabolism, fat metabolism and other reactions, which influence existence of plant life. It is present in higher amount in actively growing meristems, where it forms a part of nuclear protein. Deficiency of this element leads to a number of symptoms in plants, that include premature leaf-fall, purple or red (Anthocyanin) pigmentation in leaf, dead or necrotic areas on leaves, petioles or fruits to an overall shunting of plant growth.

In nature phosphorus occurs in the form of calcium phosphate rocks. Phosphate is available form of phosphorus, which is insoluble in the soil and is not readily transported by mass flow (Nye *et al.*, 1977). Plant absorbs it in the form of trivalent phosphate ions. One of the most important benefits of arbuscular mycorrhizal fungi is the increase in phosphorus uptake by the plant. The phosphorus absorbed by the AM fungi is either allocated directly for various structural and physiological functions of the plant or stored as polyphosphate granules in vesicles of AM fungi (Ling-Lee *et al.*, 1975; Cox and Tinker, 1976; White and Brown, 1979; Cox *et al.*, 1980; Sward, 1981; Scannerini and Bonfante, 1983). Inducible polyphosphate kinase has been implicated in translocation as well as storage of phosphorus by the fungi (Cox and Tinker, 1976; Callow *et al.*, 1978; Beever and Burns, 1980; Capaccio and Callow, 1982). Breakdown of polyphosphates probably takes place by polyphosphatase or by reversal of polyphosphate kinase, both of which have been reported from mycorrhizal plant root (Capaccio and Callow, 1982).

On genetic level one gene that has high affinity towards the phosphate called phosphate transporter has been cloned from *Glomus versiforme* (Harrison and Van Buren, 1995). The authors showed that the transported transcript are present in the external mycelium and not in the structure of the fungus internal to the host and therefore, the transporters may be responsible for the initial uptake of phosphate into the mycorrhizal system.

Role in micronutrient uptake

Micronutrients are needed by the plants in small quantities but are very important for proper growth and development, as they are parts of various enzymes, pigments and other biological molecules essential for plant life. These elements are copper, iron, zinc, magnesium and cobalt. AM fungi helps the plant in two ways: firstly, they help in the uptake of these elements, which are considered to be relatively immobile, and secondly, they uptake these elements and store them so as to prevent their concentrations to reach toxic levels. Increased uptake of iron by mycorrhizal fungi may be in part due to production

of siderophores that specifically chelate iron. Cress *et al.* (1979) found siderophore activity associated with four species of AM fungi. AM fungi are also reported to translocate sulphate from soil to the plant (Cooper and Tinker, 1978; Rhodes and Gerdemann, 1978). White and Brown (1979) suggested that polyphosphate granules could serve as a means for hyphal transporters of calcium. AM fungi act as a sink for copper, cobalt and zinc (Bowen *et al.*, 1974; Cooper and Tinker, 1978).

Role in nitrogen fixation

Nitrogen is a non-metallic element needed for formation of amino acids, purines and pyrimidines and thus indirectly involved in protein and nucleic acid synthesis. It is also a part of porphyrins and many co-enzymes of the plant system. Deficiency of this element leads to spindly growth of the plant and yellowing of leaves.

AM fungi can translocate both NH_4^+ and NO_3^- ions from soil (Ames *et al.*, 1983) and hence improve the nitrogen status of hosts in both neutral, slightly acidic and in slight alkaline soils, where ammonium and nitrate ions are predominant respectively (Smith and Gianinazzi-Pearson, 1988; Johnson and Pfleger, 1992).

Legumes are in general very responsive to mycorrhizal colonization (Schenck and Hinson, 1973; Bagyaraj *et al.*, 1979; Asimi *et al.*, 1980; Munns and Mosse, 1980). Application of rock phosphate together with AM fungi can improve nodule mass (Mosse, 1977) leading to improved nitrogen-fixation. Further, green house and field studies have shown that AM fungi improve nodulation and nitrogen fixation in legume-*rhizobium* symbiosis and actinorrhizal association (Barea and Azcon-Aguilar, 1983; Hayman, 1986). Apart from just enhancing biological nitrogen fixation, they are also involved in nitrogen transfer, since the network of AM hyphae can link different plant species growing nearby and in their nutrient uptake.

Role in carbon assimilation

The carbon requirement of mycosymbiont, is met by the plants (Ho and Trappe, 1973; Cox *et al.*, 1975). The compensation of this increased carbon demand is met by increased rates of photosynthesis in mycorrhizal plants (Allen *et al.*, 1981). Mycorrhizal plants utilize shoot carbon more efficiently than non-mycorrhizal ones (Snellgrove *et al.*, 1982). Thus, for the same leaf fresh weight and rate of photosynthesis on a leaf area basis, mycorrhizal plants have a lower percentage dry matter in their shoots and higher photosynthetic rates per unit dry matter (Snellgrove *et al.*, 1982; Smith *et al.*, 1986; Tester *et al.*, 1986).

Role in water uptake

Mycorrhizae also enhance the transport of water from soil to plant. Mycorrhizal plants have higher transpiration rates, the water uptake per unit root

length and thus, the mass flow of soil solution to the root surface is twice as compared to the non-mycorrhizal plants. Mycorrhizal (*Bouteloua gracilis*) plants closed their stomata more rapidly in response to low light or cloud flecks than non-mycorrhizal plants, thereby conserving water when light energy harvest is reduced (Allen *et al.*, 1981).

Further, a better aggregation of soil in the rhizosphere of mycorrhizal plant or in the surrounding soil induced by the hyphae could lead to a better soil water flow to mycorrhizal roots, resulting in enhanced water uptake of AM plants (George *et al.*, 1992).

The effect of AM fungal colonization and improved phosphorus nutrition include:

- Greater hydraulic conductivities
- Higher rates of transpiration under adequate soil moisture conditions
- Lower transpiration rates per unit leaf area
- Extraction of water from soil to lower water potentials
- Rapid recovery from water stress.

Role as bio-control agent

Mycorrhizal fungi do not show direct interaction with pathogen through antagonism, antibiosis or predation, but shows indirect interaction, which may be due to the physiological and morphological alteration in the host plant induced by mycorrhizal colonization (Powell, 1984). Mycorrhizal treatment significantly reduces the infection by *Rhizoctonia solani* in *Cicer arietinum* (Jalali, 1991) and root of peas caused by *Aphanomyces eutciches* (Rosendhal, 1985). Giovannetti *et al.* (1991) reported reduced severity of disease caused by *Thielaviopsis basicola* in AM-fungi inoculated tomato plant. Alfalfa seedlings inoculated with AM fungi showed reduced incidence of *Verticillium* and *Fusarium* with compared to the non- mycorrhizal ones (Hwang, 1992). The above evidences clearly indicate that AM fungi can be effectively used as bio- control agent against plant diseases.

Other group of pathogen is the nematodes, which generally affect the plant, but mycorrhizal colonization usually inhibits the growth of nematodes (Sitaramiah and Sikora, 1982; Cooper and Grandison, 1986). AM fungi control the population of plant parasitic nematodes (Kellam and Schenck, 1980; Hussey and Roncadori, 1982; Elliott *et al.*, 1984; Smith *et al.*, 1986). It has been reported that tomato infected with *Rotylenchus reniformis,* cotton infected with *Paratylenchus brachyurus*, onion infected with *Meloidogyne hapla* and *Tamarillo* infected with *Meloidogyne incognita* can be controlled by using certain AMF (Hussey and Roncadori, 1982; Sitaramaiah and Sikora, 1982; Cooper, 1984; MacGuidwin *et al.*, 1985; Cooper and Grandison, 1986). Schoenbeck and Dehne (1981) found reduced degree of infection by *Erysiphe cichoracearum* in mycorrhizal plants. *Oryza sativa* plants inoculated with *G.*

mosseae, developed resistance to the stem-rot and sheath-blight diseases caused by *Sclerotium oryzae* and *Rhizoctonia solani* respectively (Gangopadhyay and Das, 1987).

Higher amounts of amino acids, especially arginine, found in the root exudates of mycorrhizal plants, which reduces chlamydospore production in *Thielaviopsis basicola* (Dehne and Schoenbeck, 1978; Sharma *et al.*, 1992). *Glomus fasciculatum* inoculated tomato roots showed increased amount of phenylalanine and serine, being inhibitory to root-knot nematode development (Reddy, 1974). In mycorrhizal roots higher amounts of catechol is found which inhibits *Sclerotium rolfsii* growth *in vitro* (Krishna and Bagyaraj, 1986). These observations indicate that certain mechanisms involved in host resistance are activated when AM fungi infect the roots.

The mechanism involved to explain the protection from disease include (Azcon-Aguilar and Barea, 1997):

- Improved nutrient status of the host plant
- Competition for host photosynthesis
- Competition for infection sites.
- Anatomical and morphological changes in the root system
- Microbial changes in the mycorrhizosphere
- Activation of plant defence mechanism

In addition to fungal pathogens, mycorrhizae exert a strong influence on bacteria, actinomycetes, other fungi, mycoparasites and invertebrates that occur in the mycorrhizosphere (Paulitz and Linderman, 1991; Linderman, 1992; Andrade *et al.*, 1998). The interaction among AM fungi and closely associated non-pathogenic fungi, such as species of *Aspergillus*, *Gliocladium*, *Paecilomyces*, *Trichoderma* and *Wardomyces* are complex and can vary from antagonistic to neutral to synergistic (Dhillion, 1994; Mcallister *et al.*, 1996; Fracchia *et al.*, 1998; Garcia-Romera *et al.*, 1998).

Herbivory

Plants are generally affected by herbivorous insects, which destroy the plants. Generally, herbivores have either an inhibitory or neutral effect on mycorrhiza (Gehring and Whitham, 1994). A survey of literature shows that the mycorrhiza is having an intense effect on herbivorous insect. It is observed that chewing insects are negatively impacted while feeding on the mycorrhizal plants. Gange and West (1994) reported the higher concentration of anti-feedant chemicals, aucubin and catechol in mycorrhizal plants. It is hypothesized that a higher C/N ratio in the mycorrhizal plants allowed more carbon to be allocated to plant defence mechanism such as in plants secondary metabolite production.

Arbuscular mycorrhiza and phytohormones

The growth and development of plant is dependent on the secretion of growth hormones in the plant, which are also known as phytohormones. Hormone accumulation in host tissue is affected by mycorrhizal colonization with changes in the levels of cytokinin, abscisic acid and gibberellin like substances (Allen *et al.*, 1980, 1982; Barea, 1986).

The germinating spores, hyphae and secondary spores of *Glomus mosseae* independently produced two gibberellin like substances, four cytokinin-like substances and auxin-like substances (Barea and Azcon-Aguilar, 1982). Gibberellins and abscisic acid also have been reported to be increased by AM colonization (Allen *et al.*, 1982). Citrus, colonized by a presumed phosphorus tolerant *Scutellospora heterogama*, gives elevated levels of phosphorus and shows elevated levels of cytokinins in leaves, independent of phosphorus content (Edriss *et al.*, 1984).

Role in Agroecosystem

Besides improving uptake of poorly mobile nutrients (George *et al.*, 1992), AM symbiosis may also have impact on drought tolerance (Schellenbaum *et al.*, 1998) and pathogen interactions (Azcon-Aguilar and Barea, 1997) and contribute to soil quality by channelling carbon to the soil and thereby improve soil aggregation. Furthermore, there is evidence that AM fungi are important determinants of plant community structure and plant succession (Allen *et al.*, 1995). Heijden *et al.* (1998) concluded that below ground diversity of AM fungi is a major factor contributing to the maintenance of plant biodiversity and ecosystem function.

Under nutrient (Bagyaraj and Sreeramulu, 1982; Osonubi *et al.*, 1995; Beyene *et al.*, 1996) or moisture stress (Sylvia *et al.*, 1993a) conditions, AMF can significantly increase crops yield. Conventional agronomic practices may adversely affect the diversity and abundance of AM fungi in agro-ecosystem (Johnson and Pfleger, 1992; Thompson, 1994). Use of pesticides by the farmers specially fumigants, substitute aromatic hydrocarbon and benzimidazoles (Johnson and Pfleger, 1992), may adversely affect the activity of AM fungi in soil.

Arbuscular mycorrhizal fungi have a wide adaptability to certain extreme environmental conditions and this enables them to be suitable for sustainable agriculture. These fungi are known to tolerate wide range of soil pH (Daft *et al.*, 1975), soil fumigation and forest fires.

As an overall generalization one may conclude that conventional management practices reduce AM fungal populations while sustainable, organic, low-input systems tend to increase their activity (Douds *et al.*, 1993; Ryan *et al.*, 1994; Kabir *et al.*, 1998).

Role in soil aggregation

Soil aggregation is important for preventing soil loss through wind and water erosion, and the size distribution. Although soil aggregation is a complex process (Tisdall and Oades, 1982), the soil concentration of the glycoproteins, glomalin (Wright and Upadhyaya, 1996) is tightly correlated with aggregate stability across many soils (Wright and Upadhyaya, 1998). Glomalin is produced from hyphae of arbuscular mycorrhizal fungi (Wright and Upadhyaya, 1996). It is an important constituent of soil organic matter. Relatively labile carbon is protected inside the soil aggregate (Cambardella and Elliott, 1992; Jastrow and Miller, 1997; Six *et al.*, 1998), which means AMF have another indirect influence on soil carbon storage (Miller and Jastrow, 1992).

The length of the hyphae in these fungi increases with elevated CO_2 (Rillig *et al.*, 1999). Arbuscular mycorrhizal fungi produce 12-63 μg protein/mg dried hyphae (Wright *et al.*, 1996). The abundant production, apparent recalcitrance and hydrophobic characteristics indicated that glomalin might be involved in stabilization of aggregates.

This protein may contribute to soil stabilization by binding and coating aggregates and glomalin may be an easily measurable indicator of AMF activity to assess the inputs of these organisms to the agro-ecosystem (Wright and Millner, 1994).

Role in bioremediation

Arbuscular mycorrhizal fungi may play a role in bioremediation of polycyclic aromatic hydrocarbon (PAH) by establishing a plant cover in polluted soil and by modifying PAH degradation rates and pathways. One peculiarity of PAH polluted soil that may be overcome by AM plants is the hydrophobicity and resulting limitations in uptake of water and water-dissolved inorganic nutrients. The direct effect of AMF on PAH degradation would be through enhanced production of extracellular peroxidases.

Soil degradation and soil-erosion

Soil disturbance may result in physical damage to spores and/or soil hyphal network and colonized root fragments may be disrupted (Evans and Miller, 1990), altering physical, chemical and/or biological environment of the soil which prevents the colonization and germination of AM propagules which in turn reduces the microbial flora in the mycorrhizosphere (Stahl *et al.*, 1988) and results in the elimination of host plant and hence alters carbon supply to the fungus.

Several researchers have shown that inoculation of AM fungi to degraded or eroded soils results in establishment of plant species (Hall, 1980; Aziz and Habte, 1989; Jasper *et al.*, 1989). Under such soil condition AM fungal effect could be by enhancing nutrient supply to plants (Evans and Miller, 1988) and by

improving soil aggregation as the external hyphae is known to create soil aggregates by binding soil particles and possibly by producing polysaccharides (Miller and Jastrow, 1992).

Arbuscular mycorrhizae and abiotic stress

Natural and agricultural systems are often affected by adverse abiotic conditions and hence their stability is affected. AM fungi are known to play an important role not only in helping plants to tide over such stress but also to be productive (Mosse, 1986).

Water stress

Several mechanisms like alteration of leaf water potential, transpiration rates per unit length area, hormonal patterns or other physiological parameters and increased rooting length and depth are correlated to the increased drought tolerance capacity of mycorrhizal plants.

Recently, Ruiz-Lozano *et al.* (1995) found differences in proline concentration in drought stressed mycorrhizal plants and suggested that changes in the osmotic potential may contribute to their improved drought tolerance over non-mycorrhizal plants.

Salt stress

Salinity is a common problem in arid regions. Further, fertilization can lead to build up of high levels of Na^+ and Cl^- in agricultural soils (Barea *et al.*, 1983). Arbuscular mycorrhizal fungi have the ability to adapt to salt stress (Mosse, 1981) and play an important role in alleviating the detrimental effect on plants. An increase in plant tolerance to soil salinity by AM fungi is attributed to improved phosphorus nutrition (Ojala *et al.*, 1983) or by increasing potassium concentration in plant tissues which may improve the Na/K ratio in plants. Sylvia and William (1992) have attributed this role of AM fungi in alleviating ill effects of salt stress on plants on the compensation of large plant by improved nutrient acquisition.

Heavy metal stress

Role of AM fungi in alleviating the toxicity of certain metals to plants is well documented (Jeffries, 1987; Bethlenfalvay, 1992). Arbuscular mycorrhizal fungi confer resistance to plants against toxicity of cadmium, aluminium and manganese (Arines *et al.*, 1989). Retention of these ions in the rhizosphere by AM fungi could be a possible mechanism of conferring resistance.

Effect of xenobiotics/pesticides on AMF

Mycorrhizal fungi may effectively mediate and alter the interaction between plant and xenobiotic compounds by helping in the translocation of herbicides.

Uptake and translocation of the herbicide atrazine is also found in the mycorrhizal corn, which is atrazine tolerant (Nelson and Khan, 1992). Certain mycorrhizal fungi also have demonstrated the capacity to degrade herbicides atrazine and to lesser extent 2,4-dichlorophenoxyacetic acid (Donnelly *et al.*, 1993). In corn and sorghum certain herbicide safening effects by AM fungi have been found against the herbicides imazaquin, imazethapyr and pendimethalin (Siqueira *et al.*, 1991).

Conclusion and Future Perspectives

Agriculture field is of great importance in both developing and developed countries. The usage of enormous quantum of chemical fertilizers and pesticides results in the exorbitant production costs but it leads to the changes in the physical and chemical properties of the soil, which in turn has some influence on the biofertilizer and disturbs the soil.

The AM fungi have an ability to convert an arid soil into a fertile one and have a capability to resist the pests hence it plays a vital role as fertiliser and pesticides. The AM fungi may not replace use of chemicals but they will minimize their use.

Now a days, the AMF are used for the bio-hardening of the tissue culture-raised plantlets. For this, the study of symbiotic association is necessary. But AMF has not been cultured *in vitro* and that is why the study on its biochemical level is essential. The event leading to AMF colonization involves complex interaction between the plant and the fungal cells, which must be determined by the genome of both (Gianinazzi-Pearson *et al.*, 1989). Kim *et al.* (1999) have recently isolated symbiosis specific genes from *L. bicolour* that are turned on only during their interaction with the host plant. Promoters are isolated from these genes and used to construct the new generation vectors for transformation.

AMF cannot grow into the media, therefore, there must be some exchange of signals between plant and fungus after contact between their cell-wall (Becard *et al.*, 1989; Gianinazzi-Pearson *et al.*, 1990). It is now clear that there is a proper signalling between the symbiont that recognize each other by modifying their gene expression (Blair *et al.*, 1988; Berta *et al.*, 1990). The identification of these genes, which activates the fungal cell, to grow or which activate the gene of the fungal cell will be quite helpful to understand fungal plant interaction and to culture it.

A new protein appears in mycorrhizal plant roots known a endomycorrhizin which is not present in non-mycorrhizal plant root (Dumas-Gaudot *et al.*, 1984; Dumas *et al.*, 1989; Wyss *et al.*, 1990; Schellenbaum *et al.*,1992; Arines *et al.*, 1993; Simoneau *et al.*, 1994) will also be helpful in recognizing the plant fungal interaction. mRNA population in the host plant roots has recently analysed the modification in gene expression. The cloning of these genes that is functional during the two symbionts is the challenges of the future.

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CHAPTER - 10

ECTOMYCORRHIZAL SYMBIOSIS: POSSIBILITIES AND PROSPECTS

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Introduction

The dependence of forest trees on ectomycorrhizae has been recognized (Hatch, 1937; Harley and Smith, 1983). The use of specific fungi to form ectomycorrhiza on forest tree seedlings in the nursery is known to improve performance after out planting. Trees of many species especially of conifers fail to establish normally without mycorrhizal association. Artificial regeneration of species that require ectomycorrhizae fail to regenerate in the absence of ectomycorrhizae.

Most of the ectomycorrhiza have extensive multi-branching habits and hyphae from them extend considerable distance and into the soil. Trees with abundant ectomycorrhizae, therefore, have much physiologically active area for nutrient and water absorption than trees with no ectomycorrhizae. The extramaterical hyphae function as additional nutrient and water absorbing centers and increase the host's ability to capture nutrients and water from the soil. Uptake of phosphorus through mycorrhizal association in well studied and is well authenticated. Mycorrhizal association result in fast fixation of atmospheric carbon dioxide through accelerated photosynthesis to meet additional requirement of fixed carbon required for the growth and development of mycorrhizal fungus.

Ecomycorrhziae also appear to increase the tree tolerance to drought, high soil temperature, and organic and inorganic soil toxins. Ectomycorrhizae deter infection of feeder roots by some root pathogens.

The term "Mycorrhiza" is applied to associations of fungi with the absorbing organs of plants, strictly where the organ concerned is the root. The scope of the terms and the systems of nomenclature are controversial, partly due to diversity in nutritional interrelationships, in kinds of fungi and host plants involved and in morphological details of different mycorrhizas.

Traditionally, the mycorrhizas are classified as "Ectotrophic" and "Endotrophic". In ectotrophic type of mycorrhiza, the fungal endophyte is predominantly exogenous, resulting in the formation of a fungal sheath around the root. The penetration of the hyphae is normally intercellular within the host plant. The fungal partners involved in ectomycorrhiza are mostly members of basidiomycetes and few ascomycetes, infecting many tropical, subtropical and temperate forest trees. Ectomycorrhizal research in India did not get under way until the early fifties when Chaudhuri (1945) first reported mycorrhizal association in *Abies spectablis, Cedrus deodara, Morinda sp., Pinus roxburghii* and *Taxus baccata.* However, the credit for firmly establishing mycorrhizal research goes to Bakshi who claimed ecytomycorrhizal association in *Abies pindrow, Cedrus deodara, Pices morinda* and *Pinus roxburghii* (Bakshi, 1957).

Ectomycorrhizae are characterized by modification of roots and loss of absorbent hairs. The ectomycorrhizal fungus surrounds the roots and forms a mantle of mycelium. The external hyphae originating from the mantle explore the soil and help an absorption of nutrients and water, while the internal hyphae make a close contact with the roots. There are four to five thousand members of higher fungi, which form ectomycorrhizae. Ectotrophic mycorrhizal plant roots are dimorphic, uninfected long roots and infected short roots exhibiting degrees of morphogenesis and an increase in absorbing surface. The short roots become enveloped by a fungal sheath (mantle) which is well organised into structures characteristic of the fungal species. Hyphae from the sheath extend into the soil upto a few mm and many coalesce to form strands and can be traced to the fruit body of the fungus. Hyphae from the sheath extend into the roots and occupy the intercellular space in cortex as *'Hartig net'*. Nutrients from soil pass through the sheath into the cortical cells of root.

The fungus then gains carbon and other essential organic substances from the tree and in return helps the trees take up water, mineral salts and metabolites. It can also fight off parasites, predators such as nematodes and soil pathogens. Indeed, most forest trees are highly dependant on their fungal partners and in areas of poor soil, could possibly not even exist without them. Thus, in forest management, if we do not manage for the mycorrhizal fungi, we could be damaging the trees.

Ectomycorrhizal relationships are common in our forests. Most trees will only form one type of mycorrhizal relationship with fungal partners. For instance, oak, beech and birch all form ectomycorrhizal relationships with a number of fungi. This means that these trees are particularly good for finding fungi. If ectomycorrhizal fungi appear to be fruiting under these trees, they will be attached to the root tips of another tree a bit further away.

Occurrence

Ectomycorrhiza is a specialized roots organ, which is the result of a complex interaction between a plant and a compatible ectomycorrhizal fungus leading to a finely turned symbiosis (Harley and Smith, 1983). This type of mycorrhiza occurs in about 10% of the world flora, especially in trees belonging to the pinaceae (pine, larch, spruce and hemlock), fagaceae (oak, chestnut, and beech), betulceae (alder and birch), salicaceae (popular and willow), juglandaceae (hickory and pecan), myrtaceae (eucalpytus) and ericaeceae (arbutus). Some tree genera viz., *Almus, Eucalyptus, Casuarina, Cupressus. Juniperus, Tilia,* and *Ulmus* form both ectomycorrhizae and vesicular arbuscular mycorrhizae (VAM) depending on soil conditions and trees age (Hacskaylo, 1971; Marks and Kozolowski, 1973; Harley and Smith, 1983; Obase *et al.*, 2008; Tedersoo *et al.*, 2008).

More than 5000 species of fungi all over the world are known to form ectomycorrhizae. These fungi predominantly belong to basidiomycotina, and among them the mycorrhizal species belonging both to hymenomycetes and gastromycetes, in the genera *Boletus, Suillus, Russula, Cortinarius, Gomphidius, Hebeloma, Scleroderma* and *Pisolithus* (Certain ascomyectous members in Eurotiales (*Coenococcum geophilum*), Tuberales (Truffles), and Pezizales are also known to form ectomycorrhizae on trees (Ceruti and Bussetti, 1962; Kendric and Berch, 1985; Gautam and Prasad, 2001). Ectomycorrhizal fungi, therefore, have a great diversity (Oliver and Ursula, 2008; Prasad, 2008b). Out of the genera listed above, the truffles occur below ground and are dispersed where forest animals eat these and then defecate in the fecal matter (Maser *et al.*, 1978).

Spores or hyphae of the fungal symbionts when stimulated by root exudates grow vegetatively over the feeder root surface. Initially, the hyphae form a loose external weft at the point of contact with a rootlet and then penetrate outer root cells, soon forming a mantle tightly oppossed to root surface together with the harting net which surrounds outer cortical cells, but leave intact the plasmodesmata connecting one root cell to another. Ultimately, the labyrinthi ferous tissue accumulate mitochondria and golgi bodies indicating high rates of metabolic activity. Often the root cell reacts and the walls become invaginated from interaction with hartig net (Melville *et al.*, 1988; Gautam and Prasad, 2001). These alterations of root cells called transfer cells, are thought to maximize the area for exchange of materials between symbionts (O'Dell *et al.*, 1992). Thereafter, the hyphae develop intercellularly round the root cortical cells and form the hartig net which may completely replace middle lamellae between the cortical cells. Hartig net is the main distinguishing feature of ectomycorrhizae (Harley and Smith, 1983).

The fruiting bodies are seasonal, but spores of ectomycorrhizal fungi can remain dormant for undetermined length of time. Consequently, viable spores may always be available in the soil. Exudates from roots and several soil organisms can increase the spore germination of some ectomycorrhizal fungi (Fries, 1977, 1982).

Ectomycorrhizal colonization normally changes the feeder root morphology and colour. They may be unforked, bifurcate, nodular, multiforked or shaped otherwise. Their colour, which is usually determined by the colour of the mycelium of the fungal symbiont, may get black, red, yellow, brown, white or blends of these colours. Gross morphology of ectomycorrhizal roots is probably controlled by the host (Zak, 1973). O'Dell *et al.* (1992) pointed out that significance of these variations is not well established, but sometimes be inferred. Some evidence indicate that rhizomorph forming fungi improve host drought tolerance more than fungi lacking rhizomorphs (Dosskey *et al.*, 1990). Some ectomycorrhizal fungi produce substantial quantities of auxins and cytokinins, whereas others do not (Ho and Zak, 1979; Ho, 1987a, b; Ho and Trappe, 1987). Amounts and ratios of such growth regulators produced by the fungi can regulate pattern of rootlet branching and even shoot/root ratios of host seedlings, hence, differences among these fungi cause substantial differences in host morphology (Ho, 1987a). Antibiotic activity can also differ strikingly, with resultant differences in protection of rootlets from pathogens and microbial community development at mycorrhizal surface (Zak, 1976).

The functional importance of variations in structures such as mantle, branching pattern or intercellular hyphae is more difficult to interpret. Ectomycorrhizal colonization limited to the primary cortex does not spread beyond the endodermis or into meristem tissue of the feeder root (Mortin and Hilbert, 1991).

Sharma and Singh (1990) observed *Scleroderma verrucosum, S. areolatum, S. dictyosporum, Rhizopogon* spp., *Suillus* spp., *Astraeus hygrometricus, Amanita vaginala, Laccaria laccata* and *Lectarius sanguiflus* on *Pinus roxburghii* in tropical zone at an altitude of 700 m. At 1800 m. *Amanita* spp., *Lepista nuda, Lactarius sangiflus, Suillus sibricus, Boletus edulis, Scleroderma taxense, Rhizopogon rubescence* and *Thelephora terrestris* formed symbiosis with *Pinus roxburgii.*

Ectomycorrhizae: Characterization and identification

Attempts have been made to chracterize and identify the ectomycorrhizae since early twenties. Melin (1927) classified ectomycorrhizae of pines into four groups on the basis of gross morphology. Dominik (1956) incorporated many more categories, utilizing anatomical features. Both the methods were too general to give satisfactory results. Nevertheless, they provided a direction to characterization and identification of ectomycorrhizae on roots of 14 species and varieties of *Salix*. Marx (1975) characterized seven mycorrhizae on *Pinus radiatia*. Rambelli (1966) described and illustrated 10 mycorrhizae on roots of nursery seedlings of the same species. However, fungal symbionts were not identified in either of the studies. Chilvers (1968) characterized eight mycorrhizae on *Eucalyptus* and also gave identities of two fungal symbionts.

It is clear that various methods have been used by different scientists in the characterization and identification of mycorrhizae. Each method has contributed to the refinement of the techniques, though a lot remain to be done.

The ectomycorrhizae may be identified by the following characters:

1. **Morphology of ectomycorrhizae:** Whether they are simple, bifurcate, coralloid, monopodial, simple racemose, pyramidal or pinnate; the individual fork-whether elongate, short, sessile or club-shaped; colour of short roots - whether white, creamy pale-orange-brown, bright to yellow, chestnut brown or jet black; surface of mycorrhizae whether smooth, with loose weft, surface having a white crust or surface densely tomentose.
2. **Mantle characters:** Mantle structure whether pseudopar-enchymatous or prosenchymatous or palisade or loosely interwoven or lightly interwoven or duplex type; mantle thickness - which depends on virulence of the associated symbiont and physical and chemical conditions of the soil; ornamentation of mantle : hyphae emerging from mantle, mantle with setose hyphae, bristles, tortuous or dendroid structures. Hartig net- the depth of penetration of hyphae into the cortex may be usable characters.
3. Establishing mycorrhizal relationship between the roots and sporophores of fungi.
4. **Taxonomical characters**: Colour of pileus, attachment of gills, presence or absence of annulus/ volva, colour of spore deposit, size and shape of spores, their ornamentation; shape of cap-whether convex, conical, campanulate, flat, funnel shaped, umbonate or umblicate; close and outer margin-whether straight, inrolled, incurvate, undulate, lebate, or striate; surface of cap-whether smooth or glabrous, hairy, downy, tomentose, velvety, fibrillose or scaly; pellicle-separable or non-separable; annulus-wheather fixed or movable, simple or compound.
5. **Cultural characteristics**: Pure culture synthesis to confirm specific host/fungus association.
7. Chemical tests of tissues with melzer reagent and other chemicals such as potassium hydroxide and ammonium hydroxide (the two chemicals used at 2-25% for dried material and 10% for fresh material), ferrous sulphate (10%), formalin (40%), phenol (2%), aniline (pure oil or 50% in water) which give distinct colour reactions in different tissues (Singer, 1975).
8. Fluorescence of fungal tissue in long wave ultraviolet light.
9. **Microscopic characters:** Types of hyphae present, their diameter, hyphal form whether straight, undulating or dentritic, presence or absence of clamp connections and their frequency, types of branching, attached rhizomorphs - their anatomical features.

Physiology and Biochemistry

The ectomycorrhizal fungi can be isolated from short roots, spores and sporophores. Ectomycorrhizal fungi grow well in modified Melin-Norkran's medium and potato dextrose agar (PDA) medium (Bakshi, 1966). They are highly specialised in their nutritional requirements. They require soluble carbohydrates, vitamins and amino acids which they derive most, if not all, from the symbiotic niche in the primary tissues of roots.

The majority of mycorrhiza form estimated by Melin (1925) required sugars such as glucose as a source of carbon and some made fair growth on maltose, xylose and mannitol. They grew strongly on starch and inulin and no growth occured on cellulose.

It is by virtue of being specific in nutritional requirement, the ectomycorrhizal fungi colonize effectively only those substrates or soil locations where there exists either a significant free concentration of sugars or where sugars or simple organic compounds are being consistently released.

In vitro synthesis

In vitro synthesis of ectomycorrhizae studies provide the most direct and scientifically rigorous means of determining the ability of a fungal isolate to form mycorrhizae. Melin (1921, 1936) successfully demonstrated for the first time that ectomycorrhizae could be produced in synthetic cultures by inoculating seedlings of *Picea abies, Pinus sylvestris* and *'Larix europea'* with appropriate fungi. They used sand moistened with a nutrient solution as the substrate for synthesis of cultures. But sand has a relatively small surface area, a low water holding capacity and needs to be chemically cleaned for removal of undesirable inorganic and organic compounds before use in pure culture experiments.

Hacskaylo (1953) substituted sand with vermiculite in *in vitro* synthesis experiments as it is biologically sterile, void of organic material, has no constituents unfavourable for growth and the buffering capacity is only slightly greater than that of an inert material like sand. Marx and Zak (1965) further improved the substrate by stabilizing the acidity with addition of finely ground sphagnum peat moss. Marx and Bryan (1970) synthesized ectomycorrhizae of *Thelophora terrestris* and *Pisolithus tinctorius* on different conifer hosts. They observed that mycorrhizae formed by *T. terrestris* were macroscopically and microscopically different from those of *P. tinctorius*, but mycorrhizae formed by different isolates of *T. terrestris* were indistinguishable from each other regardless of the host. Marx and Ross (1970) synthesized ectomycorrhizae on *Pinus taeda* by basidiospores of *T. terrestris*. Zak (1976) described pure culture synthesis of *Arctostaphylos uva-ursi* mycorrhizae and *Laccaria laccata, Lactarius sanquiflus* and *P. tinctorius*. Zak (1976) also observed close resemblance between morphology of natural and synthesized ectomycorrhizae of Douglar fir, *Tsuga heterophylea* and *Pinus ponderosa* formed by the same fungi. Molina (1979) tested pure culturess of 28 ectomycorrhizal fungi for mycorrhiza

formation with red alder. Fortin *et al.* (1980) described synthesis of ectomycorrhiza on *Pinus strobus* seedlings within five days after inoculation with *Pisolithus tinctorius*. Duddridge *et al.* (1980) synthesized mycorrhizal rhizomorphs with aseptically germinated seedlings of *Pinus sylvestris* and *Suillus bovinus* mycelium and studied their role in water transport. Alexander (1981) described and compared ectomycorrhizae formed by *Lactarius rufus* and *Picea sitchensis* in the field and under aseptic condition. Nylund and Unestom (1982) observed the process of *in vitro* mycorrhiza formation in norway spruce using the fungus *Piloderma croceum*.

It is clear that many host and fungus species have been tested and tried the world over for establishing ectomycorrhizal synthesis and in many cases the fungi have been reisolated and their cultural characteristics have been compared with the naturally occurring ones, thus confirming and establishing their identities. But, how many of these fungi will ultimately be used on a commercial scale, as *P. tinctorius*, remains to be seen. Nevertheless, when preliminary data are obtained as all these and many more from other parts of the world, some of these would certainly prove to be useful commercially. In most of these, the host range of different species still remains to be established.

Nutrient uptake

Beneficial effects of ectomycorrhizal fungi on plant nutrition have been known (Melin, 1925; Bowen, 1973). Hatch (1937) reported that mycorrhizal white pine weighted significantly more and contained more nitrogen, phosphorus and potassium (NPK) than did non-mycorrhizal plants. Hatch (1936) observed that in the same substrate plants with mycorrhizae observed 234%, more P, 75% more K and 86% more N than plants without mycorrhizae. The role of the ectomycorrhizal fungi in the liberation of nutrients from complex compounds in the forest soil has been very much discussed (Hatch, 1937; Bjorkman *et al.*, 1967). The inverse relationship between soil fertility and ectomycorrhizal infection has long been recognised (Hatch, 1937; Bjorkman, 1942, Ducic *et al.*, 2008a.b) and ectomycorrhizal infection is often reduced by application of fertilizer (Richards and Wilson, 1963). Harley (1969, 1970) discussed the results of a number of studies of macro nutrient uptake by ectomycorrhizal plants. In general, ectomycorrhizae have been found to increase uptake of NPK but few other elements have been investigated. Ectomycorrhizae increased the Ca content of *Pinus radiata* (Henderson and Stone, 1970). Bowen (1973) pointed out the more uptake of trace elements by ectomycorrhizae trees. Bowen *et al.* (1974) observed that excised mycorrhizal roots of *Pinus radiata* absorbed more zinc than non-mycorrhizal roots. Naturally occuring ectomycorrhizal fungal symbionts in association with *P. taeda* and *P. echninata* roots were as efficient as *Pisolithus tinctorius* in the uptake of B, Cu, Fe, Mo, Mn and Zn from sewage sluge applied to nursery beds (Berry and Marx, 1976). In many observations involving mycorrhizae, the mycorrhizal association usually increased the growth of plants by enhancing the uptake of nutrients especially P (Harley and Smith,

1983; Bilgrami and Prasad, 1993; Prasad and Bilgrami, 1993; 1995, Prasad, 1995;1997a; 1997b; Gautam *et al.*, 1998). Mycorrhizal fungi in association with plant root are likely to increase P uptake by more thorough exploration of soil volume thereby making positionally unavailable "nutrients available" (Prasad, 1993). Ekwebelam (1979) investigated the effect of ectomycorrhizal fungi on the growth and nutrient uptake of caribeam pine seedlings. Nutrients content of K, Ca, Mg, Fe, B, Mn, Zn, Cu and Mo was also influenced by the ectotropic fungal symbiont (Mitchel *et al.*, 1984). Gautam and Prasad, (2001) and Prasad and Rajak (1999, 2000, 2001, 2002) reviewed the role of mycorrhizal fungi in the uptake of phosphorus by plants. Koide (1991) gave a comprehensive review on nutrient supply, nutrient demand and plant response to mycorrhizal infection. Wallander and Nylund (1991) investigated the effects of excess N on carbohydrate concentration and mycorrhizal development of *Pinus sylvestris* seedlings.

Inoculum and inoculation

Ectotrophic mycorrhizae may be initiated by several different kinds of inocula each having advantages and disadvantages in relation to the objective and economics of the inoculation programme.

Soil inocula taken from beneath the ectomycorrhizal host trees have been used extensively especially in developing countries (Mikola, 1970). Soil inoculum consists of soil already colonized by an ectomycorrhizal fungus. In bare root nurseries, upto 10% by volume of soil inoculum is incorporated to the soil before sowing. This type of inoculum has been used successfully in many cases. Gobalwin (1925) used two ounces of screened pine straw as inoculum for loblolly pine container seedling and found a significant increase in height and growth after three years. Parke *et al.* (1983) reported enhanced growth of Douglas fir seedlings inoculated with litter and humus taken from beneath Douglas fir trees.

The other type of inoculum consists of spores or macerated fruiting bodies of some ectomycorrhizal mushrooms, puffballs, or truffles. Spore inoculum is prepared by blending freshly collected fruiting bodies with tap water at high speed for two to three minutes. Some beneficial microorganisms within and on the surface of mature fruiting bodies of various ectomycorrhizal fungi have been found. Spores can be applied to the seed before sowing (Theodoroum and Bowen, 1973; Marx *et al.*, 1984; Theodoroum, 1984; Marx and Bell, 1985; Morris *et al.*, 2008; Prasad, 2008a,b) or 6-12 weeks after sowing either with a standard watering can or through the irrigation system. Spores of different fungi have been successfully used to inoculate and stimulate growth of pines in Australia (Theodoroum and Bowen, 1970, 1973; Theodoroum, 1971) and South Africa (Donald, 1975). Marx (1976, 1980) and Ruchle (1980) have had similar success with inoculating *Pisolithus tinctorious* on to assorted pine spp. in the United States. However, one of the major disadvantages of spore inoculum of most ectomycorrhizal fungi is the lack of appropriate laboratory tests to

determine spore viability. The other disadvantage is that sufficient sporophores of many fungi may not be available every year.

Another type of inoculum recommended for inoculations is pure mycelial or vegetative inoculum of ectotrophic mycerrhizal fungi (Mikola, 1973; Trappe, 1977; Marx, 1977a). Molina and Palmer (1982) studied isolation and maintenance of ectomycorrhizal pure cultures. Basically a pure culture of a particular fungus is obtained by isolating fungal material (Vegetative tissue explant) on to special media, that is than grown under aseptic conditions to produce inoculum.

Moser (1958a, b, c) was the first to make a serious attempt to produce vegetative inoculum of ectomycorrhizal fungi in Austria. For production of inoculum, mycelium of *Suillus plorans* was first grown in liquid culture than in the sterile peat moss. Takacs (1964, 1967) modified Moser's technique to produce inoculum for new pine nurseries established in formerly treeless area lacking native ectotrophic mycorrhizal fungi in Argentina.

Marx (1980) discussed in detail the early testing and development stages of producing viable inoculum of *Pisolithus tinctorius* for use in nursery and container growth seedling. Vermiculite based inoculum has been used successfully to form *P. tinctorius* ectomycorrhizae in fumigated soil on pine. Dramatic improvement in survival and growth of mycorrhizically tailored pine seedlings over naturally infected control seedling produced in containers or bare root nurseries have been reported from studies on acid coal spoils in Appalachia (Marx, 1977a, b; Marx and Artman, 1979; Walker *et al.*, 1980), Kaolin spoils in Georgia (Marx, 1977a), severly eroded site of the copper basin in Tennessee (Berry and Marx, 1978) borrow pits in South Carolina (Ruchle, 1980) and North Carolina (Goodwin, 1980) and Prairie soil (Baer and Otta, 1981).

Climatic factors

The intensity of ectomycorrhizal infection is dependent on the intensity of sunlight under which seedlings are growing. During active photosynthesis, plant roots accumulate an excess of soluble carbohydrates which, together with root exudates are necessary for the fungal symbiont to infect roots. The infection is reduced with lowering of light intensity shading of nursery beds, if made, to protect seedlings from scorching sun, should be done for a limited time at mid-day.

Ectotrophic mycorrhizae are dependent on simple carbohydrate, utilize ammonium and organic nitrogen compounds, require thiamine, other vitamins and growth factors including root exhudates, a pH in the acid range (about pH-5.0) and a temperature range of 20°C to 25°C for optimum growth. These fungi are inhibited by phenolic produces in the hymns and are sensitive to competition from saprophytes. Fertilizers added to nursery soil in high dosage have depressing effect on development of mycorrhiza. For adequate development of mycorrhiza, fertilizers should be added sparingly in the nursery. Soil

amendments including application of biocides to nursery soil initially retard mycorrhiza which, however, develops normally later on. Normal watering of nursery beds helps proper development of mycorrhiza.

Resistance to plant diseases by ectomycorrhiza feeder root pathogens such as *Phytophthora, Pythium, Rhizoctonia* and *Fusarium* infect immature and meristematic cortical tissues of roots and cause necrosis. However, one of the physiological benefits of ectomycorrhizae is the protection afforded by the fungal mantle against such root pathogens. Well formed mycorrhizal roots are resistant to infection and non-mycorrhizal feeder roots are prone to fungal necrosis even when adjacent roots have become mycorrhizal.

However, species of certain fungal genera causing ectomycorrhizae such as *Lactarius, Cortinarius* and *Hygrophorus* produce antibiotic substances while species of *Russula* produce none at all. Some of these antibiotics are antifungal on *Rhizoctonia solani, Pythium debaryanum* and *Fusarium oxysporum.* Nevertheless, it remains to be seen whether antibiotics are elaborated by ectomycorrhizal fungi *in vivo* in association with the higher symbionts. *Boletus variegatus* is known to produce volatile fungistatic compounds in pure culture. They have been identified as isobutanol and isobutyric acid. Infection of roots of *Pinus sylvestris* with *B. variegatus* resulted in the production and accumulation of volatile and fungistatic terpenes and sesquiterpenes to the extent of eight times the concentrations of such compounds in non-mycorrhizal roots.

Ectomycorrhizae : growth and development of forest seedlings

The need of many species of forest trees for ectomycorrhizal association was initially observed when attempts to establish plantations of exotic pines routinely failed until the essential fungi were introduced (Kessel, 1927; Hatch 1936; Briscoe, 1959;Van Suchtelen, 1962; Gibson, 1963). The need of pine and oak seedlings for ectomycorrhizae has also been convincingly demonstrated in the afforestation of former treeless area, such as the grasslands of Russia and the Great Plains of the United States (Hatch, 1937; McComb, 1938; White, 1941; Rosendahl and Wilde, 1942; Goss, 1960; Shemakhanova, 1962).

The concept of improving field performance of tree seedlings by forming ectomycorrhizae on them in nurseries with specific fungi ecologically adapted to the planting site was originally developed by Moser (1958a) in Austria. Using various modifications of Moser's technique and philosophy, Takacs (1967) in Argentina, Theodoroum and Bowen (1970) in Australia, and Vozzo and Hacskaylo (1971) in the United States showed experimentally that field survival and growth of tree seedlings with specific ectomycorrhizae exceeded the performance of seedlings that lacked or had few natural ectomycorrhizae at planting. Moser (1958a, b) has shown that cembra seedling artificially inoculated with pure culture of *Suillus plorans* have a better survival and initial growth after planting than the naturally inoculated seedlings. In Australia, artificial inoculation of *Pinus cembra* has now reached the stage of practical application.

Inoculation of containerized seedlings with pure cultures has been practised by many workers (Marx and Barnett, 1974; Dixon *et al.*, 1979; Maronek and Hendrix, 1979; Molina, 1980, 1982; Pawuk *et al.*, 1980; Marx *et al.*, 1982). Molina (1982) succeeded in inoculating containerized Douglas fir seedlings with four isolates of *Laccaria laccata*. Mason and Wilson (1985) have also reported improved growth of outplanted Sitka spruce seedling in pre-inoculated with *L. laccata* or *Paxillus involutus*. Marx and Cordell (1989) described the use of specific ectomycorrhiza to improve artificial forestation practices. Effective mycorrhizal fungus inoculum along with the necessary equipment and technology for successful operational application in bare root and container nurseries is now available to nursery personnel.

Ectomycorrhizae in nursery

Introduction of ectomycorrhizal fungi is also done by planting mycorrhizal (mother) seedlings in transplant beds around which seedlings are planted. The infection comes with the mother seedlings to adjoining plants which form mycorrhiza. This method has the advantage over soil inoculum in instances where soil is to be carried to long distance at the risk of loss of viability of the inoculum, in addition to other drawbacks of using soil as already mentioned earlier. This is said to be the only method of inoculating *Pinus merkusii*, which is its natural home grows well in high rainfall and low attitudinal locations in the tropics. It may appear suitable for introduction in India in similar situation where *Pinus caribaea* is growing well. *P. merkusii* may even be more promising because of its higher mean annual increment (M.A.I.) values and long fiber lengths in addition to its suitability as an industrial wood and timber over *P. caribaea*. Since it may not be possible to import mycorrhizal (mother) seedlings of *P. merkusii*, efforts may be made to introduce mycorrhiza in the species using local inocula. In this way, the mycorrhizal specificing in the species will be known. In case mycorrhiza develops the species may be raised under trial plantations.

Conclusion

Nutritional security to plants derived from ectomycorrhizal symbiosis, is known to import disease resistance in plants. In *Pinus ectinata*, mycorrhizal roots resist infection due to *Phytophthora cinnomoni*, whereas non mycorrhized roots are highly infected. This is based on the hyphothesis that the rhizosphere and the sheath surface of mycorrhizal roots possess microflora different from this in non-mycorrhizal roots. This is an area which needs further research. Non-mycorrhizal plants are more susceptible to desiccation than mycorrhizal plants. On the ground, uninfected roots present near the surface are more easily damaged by drought and frost as compared to mycorrhizal roots.

There has been increasing realization about the usefulness of mycorrhizal association in plants among botanists, agriculturists, horticulturist and foresters.

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CHAPTER - 11

HIGHER BASIDIOMYCETES MUSHROOMS AS A SOURCE OF ANTIOXIDANTS

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Introduction

Higher Basidiomycetes represent a taxonomically, ecologically, and physiologically extremely diverse group of eukaryotic organisms. Recently, extensive research on these fungi has markedly increased mainly due to their potential use in a variety of biotechnological applications, particularly for the production of food, enzymes, dietary supplements, and pharmaceutical compounds (Cohen *et al.*, 2002; Wasser, 2002). Many pharmaceutical substances with unique properties were extracted from mushrooms. The cholesterol lowering, anti-diabetic, and immunomodulating compounds are ready for industrial trials and further commercialization, while others are in various stages of development. Some of these substances are not strictly pharmaceutical products (medicines) but rather they represent a novel class of dietary supplements or nutriceuticals. The most important new pharmaceutical products from medicinal mushrooms include polysaccharides, antioxidants, and lectins (Guillot and Konska, 1997; Wasser, 2002; Ng, 2004). In the last few years, there has been significant interest in the use of mushrooms and/or mushroom extracts as dietary supplements based on theories that they enhance immune function and promote health.

Mushrooms bioactive compounds

Mushrooms have long been appreciated for their flavor and texture. Now, they are recognized as a nutritious food as well as an important source of biologically active compounds of medicinal value (Breene, 1990). Studies on new fungal compounds have been significantly expanded in the last few years. This is due mainly to the fact that fungi contain bioactive polymers able to enhance the immune system of the body, such as polysaccharides and polysaccharide-protein complexes (Wasser and Weis, 1999a,b; Wasser, 2002), as well as low-molecular-weight secondary metabolites and enzymes exhibiting direct antitumor activities (Ossowski and Lopez, 1996; Zaidman *et al.*, 2005). Such medically potent compounds have been isolated from fungal fruit bodies, mycelia, and culture liquids and have demonstrated valuable medicinal properties. Several classes of fungal biopolymers such as proteins, polysaccharides, lipopolysaccharides, and glycoproteins have been classified as molecules that have potent effects on the immune system. These compounds may restore and augment immunological responses of host immune effector cells; however, they exert no direct cytotoxicity on tumors (Rowan *et al.*, 2003). Several fungal products, mainly antioxidants, polysaccharides, and especially β-glucans were developed with clinical and commercial purposes: lentinan isolated from *Lentinus edodes* (Chihara *et al.*, 1970), schizopyllan from *Schizophyllum commune* (Komatsu *et al.*, 1969), grifolan from *Grifola frondosa* (Hishida *et al.*, 1988), krestin from *Trametes versicolor* (Sakagami and Aoki, 1991; Sakagami and Takeda, 1993), PSP also from *T. versicolor* (Yang, 1999), and many others.

Mushrooms accumulate a variety of secondary metabolites including phenolic compounds, polyketides, terpenes, and steroids. Phenolic compounds were found to have antioxidant activity (Teissedre and Landrault, 2000). These compounds have their origins as derivatives from many intermediates in primary metabolism. Some of these compounds have tremendous importance to humankind in that they display a broad range of useful antibacterial, antiviral, and pharmaceutical activities as well as less desirable toxic effects. Secondary metabolites found among fungi include substances such as antibiotics (e.g., penicillin, streptomycin), dyes (e.g., indigo), flavoring and odor compounds (e.g., menthol and limonene), and even substances such as taxol (for treatment of breast and ovarian cancers) and cyclosporin A (an immunosuppressant used to prevent transplant rejection). Some of the poisonous substances made by fungi and related to secondary metabolites, such as alpha-amanitin and phalloidin from the death cap mushrooms, muscarine from the fly agaric, orellanine from the false chanterelle, and lysergic acid from ergot of rye, have been purified and scientific and medical uses have been found as a result.

Antioxidants and their defense role

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions.

Antioxidants can scavenge free radicals and inhibit lipid peroxidation. Free radicals are defined as any molecules or atoms with one or more unpaired electrons (Yashikawa *et al.*, 1997). With the possession of the unpaired electrons, free radicals are usually unstable and highly reactive. Peroxyl radical is a key step in lipid peroxidation and is an important cause of cell membrane destruction and thus tissue damage (Halliwell, 1995). It has also been suggested that oxidative medication of low-density lipoprotein (LDL) is the main cause of atherosclerosis, which is highly related to peroxyl radical formation (Brown and Goldstein, 1983). The degenerative diseases associated with aging include cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts (Ames *et al.*, 1993). They are also associated with free radicals because oxidative damage to DNA, proteins, and other macromolecules accumulates with age and has been postulated as a major type of endogenous damage leading to aging (Fraga *et al.*, 1990). Superoxide, hydrogen peroxide, and hydroxyl radicals, which are mutagens produced by radiation, are also by-products of normal metabolism (Sies, 1986; Wagner *et al.*, 1992). Oxygen-centered free radicals and other reactive oxygen species that are continuously produced *in vivo*, result in tissue damage and cell death. Almost all organisms are well protected against free radical damage by enzymes (superoxide dismutase, catalase, and glutathione peroxidase) or compounds such as ascorbic acid, tocopherol, and glutathione. Superoxide dismutases remove O_2 by greatly accelerating its conversion to H_2O_2 and H_2O. Catalases in the peroxisomes convert H_2O_2 into water and O_2 and help to dispose of H_2O_2 generated by the action of oxidase enzymes located in these organelles (Halliwell, 1995). Tocopherols delay lipid peroxidation by reacting with chain propagating peroxyl radicals faster than these radicals can react with proteins or fatty acid side-chains. Ascorbic acid prevents aqueous oxidants from attacking and oxidizing LDL, which protects isolated human low-density lipoproteins against lipid peroxidation. It is worth noting that although living organisms possess antioxidant defense and repair systems, which have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (Simic, 1988). The presence of different oxidative agents (radiation, poisoning, and others) disturbs the balance between the system of oxidative stress and antioxidant activity, promoting the development of many diseases such as cancer, atherosclerosis, cardiovascular pathology, infections, and allergies, as well as senility. Since antioxidants are agents that suppress the oxidative stress reactions, thereby preventing or reducing oxidative damage of tissues, it was assumed that antioxidant supplements or foods containing antioxidants could be used to help the human body reduce oxidative damage. The consumption of plant foods such as fruits, vegetables, red wines, and juices provides protection against various diseases including cancer and cardio and cerebrovascular diseases (Ames *et al.*, 1993; Weisburger, 1999). This protection can be explained by the capacity of antioxidants in the plant foods to scavenge. *Te-free-radicals, which are responsible for the oxidative damage of lipids, proteins, and nucleic acids.*

There are two basic categories of antioxidants, namely, synthetic and natural. In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution, whereas natural antioxidants can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids, as well as ascorbic acid (Hall and Cuppett, 1997). Synthetic antioxidants have been used in the stabilization of foods. The most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylated hydroxyquinone (TBHQ) that are applied in fats and oily foods to prevent oxidative deterioration. However, BHA and BHT were found to be anti-carcinogenic as well as carcinogenic in experimental animals. Originally, BHA appeared to have tumor-initiating as well as tumor-promoting action. Recently, it has been established that tumor formation appears to involve only tumor promotion caused by BHA and BHT (Botterweck *et al.*, 2000).

Antioxidants from mushroom fruiting bodies

As carcinogenic properties have been reported for some synthetic antioxidants, research on the potential applications of natural antioxidants for food stabilizing against oxidation have received much attention. Screening of new, natural antioxidants is considered to be important in pharmaceutical biotechnology (Yang *et al.*, 2002). Natural antioxidants are found in plants and different groups of microorganisms, particularly in producers of melanin and other phenolic substances (Scherba *et al.*, 2001). Fungal melanin could be readily available and promising for the elaboration of new mushroom-based biotech-compounds with antioxidant activity. Some common edible mushrooms, which are widely consumed, have currently been found to possess antioxidant activity, which is well correlated with their total phenolic content. Among the four mushroom extracts from Shiitake mushroom (*Lentinus edodes*) and straw mushroom (*Volvariella volvacea*), the water extract from *L. edodes* showed the most potent radical scavenging activity, showing 75.9% (at 20 mg/ml) in the β-carotene bleaching method, 55.4% in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (at 6 mg/ml) and 94.9% of inhibition of erythrocyte hemolysis (at 5 mg/ml) (Cheung *et al.*, 2003). The ability of preparations from *Pleurotus ostreatus* (Jacq.:Fr.) P. Kumm., *Agaricus bisporus* (J. Lge) Imbach, *Lentinus edodes* (Berk.) Singer, and *Ganoderma lucidum* (Curt.:Fr.) P. Karst. in the prevention of oxidative damage of DNA has been established (Shi *et al.*, 2001; Jose *et al.*, 2002).

The mushroom *Inonotus obliquus* (Fr.) Pilat (Hymenochaetaceae) has been widely used in folk medicine in Russia, Poland, and most of the Baltic countries to improve overall health and prevent various diseases such as cancer, cardiovascular disease, and diabetes. Traditionally, *I. obliquus* has been taken in the form of a hot water extract prepared from a small piece of the mushroom (1-2 g) or one tablespoon of crushed mushroom. This produces an aqueous extract,

which is taken as a dose of three cups per day. Cui *et al.* (2005) found that *I. obliquus* exerts an antioxidant activity and protects human keratinocyte cells against oxidative stress. These authors evaluated four extracts from the fungus for antioxidant activity against the DPPH, superoxide, and peroxyl radicals. The different extracts of *I. obliquus* showed variable radical scavenging activities. The Fc extract, which contains polyphenolic components, showed strong antioxidant activity, while the Fa extract, which contained triterpenoids and steroids including lanosterol, inotodiol, trametenolic acid, and ergosterol peroxide had a relatively strong antioxidant effect. The scavenging activities of the Fa (58.7%) and Fc (81.8%) extracts of *I. obliquus* were effective at a much lower concentration (50 μg/ml). On the other hand, the Fb polysaccharide extract and the Fd extract, containing remnant polyphenolic compounds and low-molecular-weight polysaccharides, were almost inactive. The superoxide radical scavenging activity of the *I. obliquus* extracts was also generally quite high. This was especially the case for the Fc extract, whose activity was higher than that of l-ascorbic acid. Moreover, the effect of the polyphenolic extract at a concentration of 50 μg/ml was comparable to that of catalase at a concentration of 0.4 mg/ml. It is well known that polyphenolic compounds are able to efficiently scavenge superoxide radicals (Valentao *et al.*, 2002). These phenolic compounds may react with the superoxide radical via a one-electron transfer mechanism or by a hydrogen abstraction mechanism to form the corresponding semiquinone (Wang *et al.*, 1996).

The ethanolic and hot water extracts from *Hypsizygus marmoreus* were evaluated for their antioxidant properties (Lee *et al.*, 2007). In addition to the half-maximal effective concentration (EC_{50}) values in scavenging abilities on hydroxyl radicals, almost all EC_{50} values were less than 10 mg/ml indicating that these extracts were effective in assayed antioxidant properties. The major antioxidant components found in hot water extracts were total phenols (10.01-13.14 mg/g), and those in ethanol extracts were total tocopherols (33.33-10.92 mg/g).

Ear mushrooms, including *Auricularia* and *Tremella* spp., were found to be medically active in several therapeutic effects. Methanolic extracts prepared from five kinds of ear mushrooms including black, red, jin, snow, and silver ears showed moderate antioxidant activities in the 1,3-diethyl-2-thiobarbituric acid method (38.6-74.6%) at 1.0-5.0 mg/ml (Mau *et al.*, 2001). Mau *et al.* (2002) reported that the methanolic extracts obtained from several medicinal mushrooms, such as *Ganoderma lucidum* and *Ganoderma tsugae*, showed high DPPH free-radical scavenging activity (67.6-74.4%) obtained at a concentration of 0.64 mg/ml.

Antioxidants from mushroom submerged cultures

It is known that the growth rate of some medicinal mushrooms, such as *Antrodia camphorate*, in the wild is very slow, and it is difficult to cultivate in a green house, thus, it is expensive to obtain fruiting bodies (Song and Yen, 2002).

Moreover, the cultivation of other mushrooms requires 1-4 months to complete fruiting body development in solid-state fermentation. Therefore, many attempts are being made to obtain useful products from submerged mushroom cultures. This approach makes it possible to carry out directed (predominant) synthesis of the target products by selection of the appropriate culture conditions. Submerged fungus cultivation permits a fully standardized production of biomass with high nutritional value and other products with predictable composition. Therefore, using a submerged culture method to obtain useful substances from cultured mycelia might be a possible way to overcome the disadvantage of the retarded growth of fruiting bodies. Although many basidiomycetes are capable of growing in the form of mycelial biomass in submerged cultures, very little has been written so far on the antioxidant activity of submerged mycelium.

Song and Yen (2002) compared the antioxidant activity and free-radical scavenging effects of dry matter of cultural medium (DMCM), dry matter of filtrate (DMF), and different solvent extracts of mycelia from *Antrodia camphorata* in submerged culture (ACSC). The antioxidant activity of ACSC extracts was positively correlated with their ability to scavenge radicals, especially for both DMF and water extract of mycelia (WEM), which showed a potential antioxidant activity. DMCM had a lower free-radical scavenging effect, indicating that the source of antioxidant in the dry matter of the filtrate was not the original cultural medium. Authors found that the scavenging ability of dry matter of filtrate and WEM on superoxide was not correlated with their polysaccharide contents. These findings suggested that the polysaccharide content in dry matter of the filtrate and WEM was not a major factor contributing to the effectiveness of antioxidant activity. Earlier, Liu *et al.* (1997) found that polysaccharide extracts of mushrooms had scavenging effects on the superoxide that appeared to be dependent on the amount of protein present in the protein-polysaccharide complexes. Therefore, authors (Song and Yen, 2002) concluded that the polysaccharide in WEM had a higher protein/polysaccharide ratio than in dry matter of the filtrate, although no significant difference was observed in their superoxide scavenging effects. The mechanism of free-radical scavenging by polysaccharides is still not fully understood. It is only known that glycolated-protein may scavenge reactive oxygen species (ROS) by one-electron transfer or a hydrogen abstraction mechanism (Okamoto *et al.*, 1992). Thus, the protein/ polysaccharide ratio might be an important factor in ROS-scavenging ability in polysaccharide extracts. In addition, the researchers demonstrated a linear relationship between the inhibition of lipid peroxidation and the total polyphenol content (Song and Yen, 2002). Moreover, they also found high correlation between the inhibition of lipid peroxidation and the crude triterpenoids' content of non-aqueous (methanol and ethyl acetate) mycelial extracts. These results indicated that the total polyphenols in the ACSC extracts were an active component involved in the inhibition of lipid peroxidation. However, authors proposed that the triterpenoids also played a role in the non-aqueous ACSC extracts. In addition, their results indicated that the scavenging effect of crude triterpenoid was dose-dependent.

Hot-water extracts were prepared from *Agrocybe cylindracea* fruiting bodies, mycelia, and fermentation filtrate, and their antioxidant properties were studied (Tsai *et al.*, 2006). The yields of extracts were in the descending order of filtrate, fruit bodies, and mycelia. The higher yield of filtrate was mainly due to the fact that most components contained in the filtrate were small and readily water-soluble. *It is worth noting that the yields of the obtained in these experiments' hot-water extracts from fruit bodies, mycelia, and filtrate were higher than those of the methanolic extracts* (33.5%, 28.0% and 28.0%, respectively) (Huang *et al.*, 2002). Antioxidant activities of hot-water extracts from fruit bodies, mycelia, and filtrate were 63.6%, 81.6%, and 56.8% at 20 mg/ml, respectively. EC_{50} values in reducing power were 2.72, 3.97, and 3.09 mg/ml, respectively, whereas those in scavenging abilities of 1,1-diphenyl-2-picrylhydrazyl radicals were 0.62, 1.66 and 0.82 mg/ml for fruit bodies, mycelia, and filtrate, respectively. At 20 mg/ml, the scavenging abilities of hydroxyl radicals were 80.1%, 57.0%, and 54.3% for fruit bodies, mycelia and filtrate, respectively. With regard to EC_{50} values in chelating abilities on ferrous ions, the hot-water extract from filtrate was better than that from mycelia. Total phenols were the major naturally-occurring antioxidant components found in hot-water extracts and in the range of 23.74–30.16 mg/g. From EC_{50} values obtained, it can be concluded that hot-water extracts from three forms of *A. cylindracea* were effective in antioxidant properties. It is worth noting that the naturally-occurring antioxidant components, including ascorbic acid and total phenols, were found in hot-water extracts from fruit bodies, mycelia, and filtrate. Tocopherols were found in hot-water extracts from fruit bodies and mycelia. Total phenols were the major naturally-occurring antioxidant components found in hot-water extracts from *A. cylindracea* and in the range of 23.74–30.16 mg/g. Total antioxidant components varied among hot-water extracts and were 30.46, 27.72, and 24.57 mg/g for fruit bodies, mycelia and filtrate, respectively. Authors emphasized that the high content of total phenols in all hot-water extracts might explain high-antioxidant properties in *A. cylindracea*.

Mau *et al.* (2005) investigated the antioxidant properties of *Ganoderma* species. Generally, the production of *Ganoderma tsugae* Murrill and *G. lucidum* (W. Curt.:Fr.) P. Karst., call Ling chih or reishi, includes a long-time cultivation in plastic bags for fruit bodies, and a short-time submerged fermentation for mycelia and fermentation filtrate. Normally, mature Ling chih is harvested from plastic bags at 1-2 months after fruiting, whereas baby Ling chih is harvested at 2-3 weeks after fruiting. Mycelia and its fermentation filtrate, prepared from submerged culture, are alternative or substitute products of mature and baby Ling chih. Hot-water extracts from four forms of *G. tsugae* (mature and baby Ling chih, mycelia, and fermentation filtrate) were prepared, and their antioxidant properties were compared. Hot-water extracts from mature and baby Ling chih showed high antioxidant activities (78.5% and 78.2%) at 20 mg/ml, and had EC_{50} values of 7.25 and 5.89 mg extract/ml, respectively. EC_{50} values in reducing power were 1.12, 1.37, 2.48, and 1.41 mg extract/ml, whereas those with scavenging abilities of DPPH radicals were 0.30, 0.40, 0.72 and 5.00 mg

extract/ml for Ling chih, baby Ling chih, mycelia, and filtrate, respectively. At 20 mg/ml, scavenging abilities on hydroxyl radicals were in descending order of Ling chih>baby Ling chih>mycelia>filtrate. Naturally-occurring antioxidant components, including ascorbic acid, α- and δ-tocopherols, and total phenols were found in hot-water extracts from fruit bodies, mycelia, and filtrate. Total phenols were the major naturally-occurring antioxidant components found in hot-water extracts from *G. tsugae* in the range of 40.86–42.34 mg/g. However, β-carotene and γ-tocopherol were not detected in the hot-water extracts since they are fat-soluble. Total antioxidant components varied among hot-water extracts and were in descending order of baby Ling chih (44.72 mg/g) Ling chih (44.62)>mycelia (41.85) ± filtrate (41.53).

The same researchers (Mau *et al.*, 2004) evaluated the antioxidant properties of methanolic extracts from three mushroom mycelia: *Grifola frondosa* (maitake), *Morchella esculenta* (morel), and *Termitomyces albuminosus* (termite mushroom). Methanolic extracts from the three mycelia showed high antioxidant activities (85.4–94.7%) at 25 mg ml^{-1}. Reducing powers of the three methanolic extracts were 0.97–1.02 at 25 mg ml^{-1}. Scavenging effects on DPPH radicals were 78.8–94.1% at 10 mg/ml^{-1}. These three mycelia showed no scavenging effect on hydroxylradicals. Chelating effects on ferrous ions were high (90.3–94.4%) at 10 mg ml^{-1}. Total phenols were the major naturally-occurring antioxidant components found in methanolic extracts. Contents of ascorbic acid and tocopherols were similar for these three mycelia. All EC50 values were below 10 mg ml^{-1}, indicating that the three mycelia had good antioxidant properties except for the scavenging effect on hydroxyl radicals.

The ethanolic and hot-water extracts from fruit bodies and mycelia of white mutants of *Hypsizygus marmoreus* were prepared and compared (Lee *et al.*, 2008). Using the conjugated diene method, fruit bodies showed higher antioxidant activities than mycelia. At 5 mg/ml, antioxidant activities of ethanolic and hot-water extracts from fruit bodies (53.3% and 65.6%) were higher than those from mycelia (24.5% and 32.6%, respectively). At 20 mg/ml, antioxidant activities of ethanolic and hot-water extracts from fruit bodies (98.6% and 75.4%) were higher than those from mycelia (87.1% and 74.6%, respectively). However, antioxidant activities were 100% and 97.1% at 0.1 mg/ml for BHA and α-tocopherol, respectively, and 79.8% at 10 mg/ml for ascorbic acid. It is interesting that at 5 mg/ml, ethanolic and hot-water extracts from the fruit bodies of a normal strain of *H. marmoreus* exhibited moderate antioxidant activities of 56.4% and 38.6%, respectively (Lee, 2003). The authors suggested that the antioxidant activity of extracts might be due to the reduction of hydroperoxide, inactivating free radicals, or forming complexes with metal ions, or combinations thereof. When studying scavenging ability, ethanolic extracts showed higher scavenging abilities on DPPH radicals than hot-water extracts. At 5 mg/ml, scavenging abilities were 75.5% for both ethanolic extracts and 36.8% and 55.5% for hot-water extracts from fruit bodies and mycelia, respectively. At 10 mg/ml, scavenging abilities were 94.8-96.5% for ethanolic extracts and 40.3% and

81.8% for hot-water extracts from fruit bodies and mycelia, respectively. However, at 0.1 mg/ml, scavenging abilities of BHA and α-tocopherol were 94.9% and 93.5%, respectively, whereas that of ascorbic acid was 27.2-35.4% at 1-20 mg/ml. The scavenging ability of the ethanolic and hot-water extracts at 5 mg/ml from a normal strain of *H. marmoreus* fruit bodies on DPPH radicals were 59.7% and 44.2%, respectively (Lee, 2003). At 10-20 mg/ml, two different patterns were found on scavenging abilities, which were 92.4-93.2% and 64.1-77.2% for ethanolic and hot-water extracts, respectively (Lee, 2003). Authors concluded that the ethanolic extract from the white mutant was more effective in scavenging ability on DPPH radicals than that of the normal strain whereas hot-water extracts from both strains were comparable. However, the better ability of ethanolic extracts might be due to more hydrogen-donating components extracted by ethanol.

Effectiveness of antioxidant properties inversely correlated with their EC_{50} values. With regard to EC_{50} values of antioxidant activities by the conjugated diene method, hot-water extracts were more effective than ethanolic extracts, whereas fruit bodies were more effective than mycelia (Lee *et al.*, 2008). Effectiveness in reducing powers was in a descending order: hot-water extract from mycelia ethanolic extract from fruit bodies > ethanolic extract from mycelia>hot-water extract from fruit bodies. With regard to the scavenging ability on DPPH radicals, EC_{50} values of the extracts were 2.85-4.36 mg/ml, except for that of the hot-water extract from fruit bodies (18.85 mg/ml). Various extracts were less effective in scavenging abilities on hydroxyl radicals, and EC_{50} values were 31.74-89.34 mg/ml. However, hot-water extracts were more effective than ethanolic extracts. Generally, all EC_{50} values of 10 mg/ml indicated that various extracts were effective in these antioxidant properties. In addition to EC_{50} values in scavenging abilities on hydroxyl radicals, almost all EC_{50} values were less than 10 mg/ml, indicating that the extracts were effective in these assayed antioxidant properties. However, EC_{50} values of the hot-water extract from fruit bodies in the scavenging ability on DPPH radicals and the ethanolic extract from fruit bodies in the chelating ability on ferrous ions were 18.85 and 15.86 mg/ml, respectively. As compared to the normal strain of *H. marmoreus* fruit bodies, the white mutant was more effective than the normal strain in antioxidant activity and reducing power. Furthermore, ethanolic extracts from the white mutant were more effective than those from the normal strain in scavenging abilities on DPPH and hydroxyl radicals, and the hot-water extract from the white mutant was more effective than that of the normal strain in chelating ability on ferrous ions.

When the antioxidant components were studied, naturally-occurring tocopherols and total phenols were found in four extracts from *H. marmoreus* fruit bodies and mycelia (Lee *et al.*, 2008). Ascorbic acid was not detected due to the fact that it is easily degraded by heat. However, β-carotene was only found in the ethanolic extract from mycelia. Total phenols were the major antioxidant components found in hot-water extracts, whereas total tocopherols were the

major antioxidant components found in ethanolic extracts due to its fat-soluble nature. Contents of tocopherols were 33.33 and 10.92 mg/g for ethanolic extracts from fruit bodies and mycelia and 0.28 and 3.25 mg/g for hot-water extracts, respectively. Due to the discrepancy in the profile of contents of total tocopherols and total phenols, these four extracts showed different effectiveness in antioxidant properties assayed. The contents of total assayed antioxidant components were 40.22 and 18.01 mg/g for ethanolic extracts from fruit bodies and mycelia and 10.29 and 16.39 mg/g for hot-water extracts, respectively. Correlations of contents of total antioxidant components and total tocopherols (plus β-carotene) with EC_{50} values of antioxidant activity, reducing power, scavenging ability on DPPH radicals, and chelating ability on ferrous ions were established, whereas the correlation of total phenol content was established only with that of the scavenging ability on hydroxyl radicals. Authors conclude that the contents of total phenols and tocopherols in these extracts were responsible for their effective antioxidant properties. Moreover, although BHA, ascorbic acid, and/or α-tocopherol have significant antioxidant activity, reducing power, and scavenging ability on DPPH radicals, they are additives and are used or are present in mg levels in foods. Various extracts of *H. marmoreus* could be used in grams or hundreds of gram levels as food or a food ingredient. Therefore, mushrooms in human diets might provide health protection to help humans reduce oxidative damage daily.

Occurrence of the antioxidant and free-radical scavenging activity among higher Basidiomycetes mushrooms

The analysis of literature data shows that surprisingly scarce information exists on the higher Basidiomycetes antioxidant activity in submerged cultivated mycelium. The most comprehensive study was done by Badalyan (2003). The mycelial samples (cultured liquid, mycelial extract, and biomass suspension) of 14 mushroom cultures (*Coprinus comatus*, *C. disseminatus*, *C. micaceus*, *Hypholoma fasciculare*, *Lentinus edodes*, *Lepista personata*, *Marasmius oreades*, *Pholiota alnicola*, *Pleurotus ostreatus*, *Stropharia coronilla*, *Suillus luteus*, *Schizophyllum commune*, *Trametes versicolor* and *Volvariella bombycina*) tested possessed certain antioxidative potential to inhibit the reaction of free-radical peroxide oxidation of lipids in rat brain homogenate (Badalyan, 2003). The level of observed antioxidant activity depended on the bio-ecological differences of tested strains (geographical origination, type of wood substrate, mycelial growth rate, and morphology), as well as the experimental conditions. Mycelia of seven screened species (*Pholiota alnicola*, *Lepista personata*, *Trametes versicolor*, *Volvariella bombycina*, *Schizophyllum commune*, *Suillus luteus*, and *Lentinus edodes*) showed more than 20% antioxidant activity.

Recently, we screened twenty-eight higher Basidiomycetes strains belonging to various taxonomic and ecological groups for their antioxidant (Asatiani *et al.*, 2007a) and free-radical scavenging activity (Asatiani *et al.*, 2007b) after submerged cultivation in the synthetic medium of simple

composition. No correlation was revealed among fungi belonging to different ecological groups, but the antioxidant activity (AOA) of the extracts significantly depended on mushroom species. Water extracts from *Coprinus comatus* 306, *Agaricus nevoi* 408, and *Flammulina velutipes* 104 in concentration of 2 mg/ml manifested very high AOA (more than 85%), whereas the water extracts from *Daedalea gibbosa* 514, *Pleurotus citrinopileatus* 435, and *Macrolepiota excoriata* 519 at the same concentration showed very low AA. When the ethanol extracts were tested, the highest values of AOA were received with *Agaricus nevoi* 408 samples followed by *Omphalotus olearius* 1079 and *Auricularia auricula-judae* 1036, 92.1%, 83.4%, and 80.2%, respectively, at an extract concentration of 2 mg/ml. In contrast to these fungi, no antioxidant activity was exhibited in *Coprinus comatus* 906 extract at the same concentration, while *Phellinus robustus* 531 showed only 17.6% of inhibition. The comparison of the antioxidant potential of extracts received from mushroom biomasses with two different solvents elucidated that the water extracts of *Leucoagaricus leucothites* 1075 and *Phellinus robustus* 531 had higher activity than their ethanol extracts. However, the ethanol extracts received from the mycelial biomasses of *Daedalea gibbosa* 514, *Pleurotus citrinopileatus* 435, and *Trametes versicolor* 1013 showed higher antioxidant activity compared with the water extracts (Table 11.1).

Table 11.1. Antioxidant activity (%) of water (culture liquid) extracts from dried submerged mushroom mycelia

Species	Culture liquid (water) extracts			Ethanol extracts		
	Extract concentration (mg/ml)					
	2.0	4.0	8.0	2.0	4.0	8.0
Agaricus nevoi 408	86.9	88.7	89.6	92.1	93.7	95.0
Coprinus comatus 306	88.6	82.7	90.8	72.6	82.2	88.8
Coprinus comatus 906	26.0	31.9	46.3	-2.4	39.2	62.1
Daedalea gibbosa 514	16.3	43.5	65.0	36.7	65.6	79.9
Flamullina veluthipes 104	85.3	89.9	90.9	77.3	83.6	87.1
Leucoagaricus leucotites 1075	63.9	79.9	83.1	51.5	62.1	57.6
Pleurotus citrinopileatus 435	22.5	6.2	5.2	37.0	38.1	50.6
Phellinus robustus 531	38.8	63.2	68.5	17.6	44.9	71.1

The free-radical scavenging activity was also species-dependent. The highest activity at a minimal sample concentration of 0.5 mg/ml was shown with water extracts from *Ganoderma lucidum* 545 (69%) and *Daedalea quercina* 943 (49%), whereas mycelial biomasses of *Pleurotus citrinopileatus* 435, *Stereum hirsutum* 524, and *Pleurotus nebrodensis* 1019 showed very weak scavenging ability toward DPPH, only 11% (Table 11.2).

Table 11.2. Scavenging ability (% of inhibition) and EC_{50} values of water and ethanol extracts from the submerged mushroom mycelia

Species	Water extract (mg/ml)				EC_{50} (mg/ml)
	0.5	1.5	3.0	9.0	
Daedalea quercina 943	49±2.3	62±2.8	65±2.6	39±1.8	0.7±0.1
Ganoderma lucidum 545	69±1.6	70±1.8	60±1.5	32±2.5	2.4±0.3
Pleurotus citrinopileatus 435	11±2.4	26±3.3	31±4.2	38±2.1	>9
Pleurotus nebrodensis 1019	11±2.1	9±1.5	13±3.1	12±2.6	6.7±0.9
Stereum hirsutum 524	11±3.3	23±3.6	45±5.7	61±3.0	3.3±0.9
	Ethanol extracts (mg/ml)				
	0.5	1.5	3.0	9.0	
Flammulina velutipes 1046	31±1.9	50±2.9	55±1.8	49±1.4	1.6±0.4
Ganoderma lucidum 545	56±2.2	77±1.9	68±2.4	49±2.2	2.2±0.3
Leucoagaricus leucotites 1075	20±2.3	31±1.5	26±2.6	7±1.6	7.3±0.9
Omphalotus olearius 1079	39±1.9	62±2.1	65±1.6	58±2.2	0.9±0.1
P. citrinopileatus 435	9±1.6	27±3.0	46±2.1	68±2.7	3.8±0.4

For the majority of screened mushrooms, the sample concentration of 1.5 mg/ml was sufficient to exhibit maximal scavenging activity. This concentration appeared to be much lower than that in experiments with wild edible mushrooms (Ferreira *et al.,* 2007) and quite comparable to those shown for mycelium of *Ganoderma tsugae* (Mau *et al.*, 2005). The highest scavenging effect expressed the water extract from *Flammulina velutipes* 1046 at a sample concentration of 3.0 mg/ml, 85% of inhibition, which was close to the BHA standard. The scavenging effect of ethanol extracts ranged from 1% (data not shown) (*Stereum hirsutum* 524, *Trametes zonata* 450) to 56% (*Ganoderma lucidum* 545) at a sample concentration of 0.5 mg/ml. In accordance with other published data, the scavenging effects of ethanol extracts obtained from mushroom submerged mycelia increased when concentration increased (Mau *et al.,* 2002; Cheung *et al.,* 2003; Ferreira *et al.,* 2007). The scavenging ability of extract from *G. lucidum* 545 and *Pleurotus cystidiosus* 225 increased by 21 and 27%, respectively, with the sample concentration augmentation to 1.5 mg/ml. Such a sample concentration was sufficient to reveal maximal scavenging ability of the majority of ethanol extracts tested. The scavenging activity of *P. citrinopileatus* 435 and some other fungi significantly improved with the gradual elevation of sample concentration from 0.5 to 9.0 mg/ml.

Conclusions

This study shows that the capability to accumulate the high levels of antioxidant compounds is widespread among higher Basidiomycetous fungi. Moreover, the submerged mycelium of most investigated mushroom species possess high AOA and radical scavenging potentials and may serve as a good,

easily accessible, low-cost source of safe, natural antioxidants. However, in spite of the fundamental and commercial importance of higher Basidiomycetes only a few species' fruiting bodies and submerged mycelia have been comparatively evaluated for their antioxidant activity. No data exist on the fundamental factors affecting the synthesis of antioxidants in submerged and solid-state fermentation of lignocellulose, especially by the ligninolytic white-rot fungi. Further studies are needed to determine the physiological mechanisms regulating antioxidant accumulation, to isolate active component(s), and to establish the pharmacological efficacy of promising mushroom extracts.

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CHAPTER - 12

THE CONTRIBUTION OF WILD FUNGI TO DIET, INCOME AND HEALTH: A WORLD REVIEW

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Trends in consumption of wild fungi around the world

Wild fungi are a natural resource widely acknowledged for their nutritional and economic value and their medicinal properties. About 1200 species are recorded to be used in eighty-five different countries (Boa, 2004). The first record of fungi being eaten comes from China, about 900 B.C. (Aaronson, 2000). Nowadays, the trends in fungi consumption vary from region to region.

Europe

People from eastern countries and Russia have a stronger tradition of collecting fungi from the wild than people from western, northern, and central parts of the continent (Wasson and Wasson, 1957; Druzhinina and Palma-Oliveira, 2004). Historically, Germanic populations have never been great fungi eaters, in contrast to Romans and Slavs (Kardell, 1980). A good example for this general trend can be found in Scandinavia. In Sweden the custom of eating fungi was imported from France, and today more than 50% of the population gathers fungi for consumption (Kardell, 1980). In Finland there are two traditions of fungus use: One came through France and Sweden to the educated people of southwest Finland; the other came from the east via Karelia and was adopted by ordinary country folk. The western tradition favoured chanterelles and boletes, the eastern acrid *Lactarius* (Härkönen, 1998). Another example of these cross-cultural differences in mushroom appreciation can be found in the Bering Strait: wild mushrooms are highly esteemed in the Russian side while being feared and avoided in Alaska (Yamin-Pasternak, 2008).

In the UK, the consumption of wild fungi has always been considered of minor importance. Extrapolating the results of a survey carried out by Barnett *et al.* (1999), the average intake rate could be 0.12 kg of fresh weight per person per year, and in most cases only one species is consumed, generally *Agaricus campestris*. Another survey by Byrom *et al.* (1995) reported that only 37.3% of adults consumed fungi (cultivated and wild species) throughout the UK.

In Spain, Basques and Catalans were always eager mushroom consumers, but now interest in picking and eating wild fungi has spread over the country, and people from other regions where there was, traditionally, no interest in wild fungi are looking forward to the mushroom season each year (De Roman and Boa, 2004). In Portugal there is a long tradition of fungus consumption, and the most appreciated species are *Leccinum corsicum* and *Macrolepiota procera* (Martins, 2004). In a survey carried out by Pereira *et al.* (2001), 74% of the people interviewed affirmed they consumed fungi.

The most appreciated wild mushrooms in Italy nowadays are the porcini (*Boletus* spp.) followed by *Cantharellus cibarius*, *Amanita caesarea*, *Calocybe gambosa*, *Morchella* spp., *Armillaria mellea* and *Lactarius deliciosus* (Sitta and Floriani, 2008). Truffles are very important economically, especially the white Alba truffle (*Tuber magnatum*) and the Norcia black truffle (*Tuber melanosporum*).

The French have always had an eye for culinary delicacies. Nowadays there are so many people interested in picking wild fungi that some measures had to be taken in some regions: establishment of legal regulations of the harvest of wild fungi; introduction of a license system for mushroom pickers; distribution of leaflets and posters to raise public awareness about good harvesting practices and the threats of overexploitation (Berelle, 2003).

Africa

Fungi are in general highly appreciated as food stuffs and considered to be similar to meat. They appear at the beginning of the rainy season when the crops from the previous season are almost finished and the new crops have just been planted (Härkönen 2002). Several general tendencies can be observed across the continent: Boletes are nowhere appreciated, and the species of the genus *Agaricus* are considered inedible. It is also worth to note that some species which are appreciated nowhere outside Africa are of current use, for example *Schizophyllum commune* and polypores (Rammeloo and Walleyn, 1993).

Several ethnomycological studies have been carried out in eastern and southern Africa. In Zambia *Termitomyces* species are among the most preferred fungi, especially *T. letestui*. Other fungi regularly consumed are *Lactarius kabansus*, *Cantharellus cibarius*, *C. pseudocibarius*, *C. densifolius*, *C. longisporus*, *C. miniatescens* and *Amanita zambiana* (Pegler and Piearce, 1980; Degreef *et al.*, 1997; Masuka, 2002).

In Zimbabwe a household consumes 5-20 kg of wild fungi per year. *Amanita zambiana* is the most sought after. *Termitomyces* species are also highly rated, with *T. clypeatus*, *T. eurhizus*, *T. microcarpus* and *T. titanicus* being the most liked. *Cantharellus cibarius*, *C. pseudocibarius*, *C. longisporus* and *C. miniatescens* are also considered as delicacies. *Lactarius* (*L. kabansus*, *L. edulis*, *L. inversus*) and *Russula* (*R. cellulata*) species are also consumed (Masuka, 2002).

In northern Mozambique about 72-160 kg of wild fungi are consumed per household per year. *Termitomyces schimperi* represents about 50% of the fungi consumed. Other appreciated species are *Amanita zambiana*, *A. loosi*, *Cantharellus cibarius*, *C. symonsii* and *Lactarius kabansus* (Masuka, 2002).

Malawians have a keen interest in, and an extensive knowledge of, a wide variety of edible fungi. The main edible fungi belong to 3 genera: *Termitomyces* (*T. microcarpus* is a delicacy), *Cantharellus* (*C. cibarius*, *C. congolensis* and *C. longisporus*) and *Amanita* (*A. zambiana*, *A. goossensiae* and *A. bingensis*) (Morris, 1984; Masuka, 2002).

In Tanzania there are large differences between tribes, but the majority of people like fungi very much. People living in mountainous areas prefer wood-inhabiting fungi, and those living in miombo woodlands prefer mycorrhizal fungi, but in both areas *Termitomyces* species are considered the best, especially *T. letestui*, *T. eurrhizus*, *T. microcarpus* and *T. singidensis* (Härkönen, 2002; Masuka, 2002). Some brightly coloured *Amanita* species are also well liked by Tanzanians, including *A. mafigensis*, *A. hemibapha* and *A. tanzanica* (Härkönen *et al.*, 1994).

In Zaire, *Cantharellus* species occupy the first place regarding the number of species and quantity harvested. These are followed by *Amanita* and *Termitomyces*. Local inhabitants show an innate knowledge about fungi since no cases of poisoning are known (Parent and Thoen, 1977).

In Ethiopia the favourite fungus is *Schizophyllum commune*, followed by a *Lentinus* sp. similar to that collected and consumed in some countries in Southeast Asia like Thailand, Lao P. D. R. and Indonesia (Tuno, 2001).

In Madagascar, *Russula* spp. and *Cantharellus* spp. are the most widely consumed and economically important mushrooms (Buyck, 2008). *Suillus* spp. are mainly canned for export but also found in markets in Antananarivo, and *Rhizopogon* cf. *luteolus* is consumed locally. *Clavulina albiramea* and *Afroboletus luteolus* are also eaten but of lesser importance. Wood-inhabiting species are occasionally gathered for consumption, such as *Tremella fuciformis*, *Collybia aurea*, *Auricularia* spp., *Oudemansiella* spp., *Lentinus* spp. and *Pleurotus* spp. It is interesting to note that the genus *Termitomyces*, so common in the mainland, is absent from Madagascar.

On the contrary, little ethnomycological research has been undertaken in western Africa. Ogundana (1978) found that the most popular mushroom with Nigerians is *Termitomyces robustus*. Other species eaten are *T. globulus*, *T.*

microcarpus, *Tricholoma lobayensis* and *Pleurotus squarrulosus*. Two Nigerian tribes, the Igbo and the Yoruba, have also been the subject of ethnomycological studies (Oso, 1976; Akpaja *et al.*, 2003).

A study carried out among two ethnic groups of Cameroon (Van Dijk *et al.*, 2003) revealed that fungal knowledge was extensive but the consumption was low, averaging 1.1-1.4 kg of fresh fungi per person per year, a rate that is much lower than in central and eastern Africa. This might have to do with the poor social valuation of fungi among these groups. The most appreciated species are *Termitomyces* spp., *Marasmius katangensis*, *Lentinus squarrulosus*, *Cantharellus rufopunctatus* and *Volvariella volvacea*.

Yorou and De Kesel (2001) found that the Nagot people of Benin eat about 50 species of wild fungi, but they only play an important role in their diet during the rainy season, when meat is scarce. The most appreciated species are the big ones, especially *Termitomyces*, but other species such as *Psathyrella tuberculata* are also relished.

In Senegal, people living in the forested areas consume fungi in large quantities, whereas people of the savannas do not like them. Religion might explain these differences: forest people have been mainly converted to Catholicism, savanna people to Islam (Rammeloo and Walleyn, 1993).

In Burkina Faso, the gathering and consumption of mushrooms seems to be dying out due to declining mushroom populations resulting from disappearing forest habitats. A study among three ethnic groups revealed that they eat 24 mushroom species, and the most preferred are *Chlorophyllum* aff. *molybdites* and *Phlebopus sudanicus* (Guissou *et al.*, 2008).

In northern Africa the most appreciated fungi are the desert truffles, belonging to the genera *Terfezia* and *Tirmania* (Trappe, 1990). Other highly valued species of desert truffle is *Kalaharituber pfeilii*, consumed by the Khoisan people of the Kalahari Desert in southern Africa (Trappe *et al.*, 2008).

Asia

The Chinese and Japanese are particularly enthusiastic fungi consumers. Matsu-take (*Tricholoma matsutake*), Shiitake (*Lentinula edodes*), Shimeji (*Hypsizygus tessulatus*), Enoki-take (*Flammulina velutipes*) and Nameko (*Pholiota nameko*) are the preferred fungi in these latitudes (Pegler, 2000, 2003). An ethnomycological survey by Härkönen (2002) in the Hunan province of China revealed that everybody uses fungi for food. The most appreciated fungus there is indeed a lichen, *Umbilicaria esculenta*, and species from the genera *Pleurotus*, *Lentinus*, *Auricularia*, *Ramaria*, *Lactarius*, *Russula* and *Amanita* are also widely appreciated.

In India, mushrooms are consumed by the tribal and rural population during the monsoon season (Kulkarni and Dighe, 2003), although this tradition is on the decline because of growing urbanisation and the associated changes in food habits (Agrahar-Murugkar and Subbulakshmi 2005). About 70 species of edible

mushrooms can be found in Himachal Pradesh (Das *et al.*, 2002). Harsh *et al.* (1996 and 1999) reported that tribal people of Madhya Pradesh eat *Astraeus hygrometricus*, *Calvatia cyathiformis*, *Geastrum fimbriatum*, *G. triplex*, *Lycoperdon pusillum*, *Mycenastrum corium*, *Podabrella microcarpa*, *Termitomyces heimii* and *Scleroderma radicans*.

In Nepal, all castes and ethnic groups consume fungi, except for the Brahmans, for whom it is forbidden (Adhikari and Durrieu, 1996). Christensen *et al.* (2008) reported 228 mushroom species collected for consumption, among which *Laetiporus sulphureus* is the most widely collected. Several species are used exclusively in Nepal, such as *Lactarius thakalorum*, *Amanita chepangiana*, *Laccaria vinosoavellana*, *Lactarius subpiperatus* and *Polyporus badius*. It is interesting to note that *Gyromitra infula* and *Pholiota squarrosa* are commonly eaten in Nepal but considered poisonous or non-edible elsewhere. In Afghanistan and Pakistan people restrict the consumption of fungi to morels and gasteromycetes, which are usually sold in dried form (Batra, 1983).

Many wild fungi are available at markets in Thailand, but *Termitomyces* species are by far the most appreciated delicacies (Jones *et al.*, 1994). There is little information regarding the use of fungi in the rest of Southeast Asia. Sather (1978) reported that an Indian folk in Malaysia, the Iban, eat varieties of at least 25 genera of fungi, but this use is extensive rather than intensive, because fungi contribute very little to their diet.

Australia and the Pacific

In Australia aborigines consume *Choiromyces aboriginum* (a highly prized native truffle), *Cyttaria gunnii*, *Mycoclelandia bulundari*, *Piptoporus* sp., *Pisolithus tinctorius*, *Polyporus mylittae*, and an unidentified species called Mulga Bolete (Kalotas, 1997). Trappe *et al.* (2008) also report aborigine consumption of other desert truffles, such as *Elderia arenivaga*, *Mycoclelandia arenacea*, *M. bulundari* and *Mattirolomyces mulpu*, but traditional knowledge is endangered and desert truffles have currently little value for contemporary aboriginal communities.

In the Fiji Islands natives are in general suspicious of fungi. This attitude does not seem to have been introduced by the English because all the local words for fungal fruitbodies are true Fijian words and not derived from the English. Only *Auricularia* sp. is widely collected and used as food (Markham, 1998). For the Nuaulu people on the island of Seram (eastern Indonesia) mushrooms have only a marginal importance as food or for hallucinogenic purposes (Ellen, 2008).

America

In North America, most of the wild fungi that are harvested are not for personal consumption, but for sale. The Pacific Northwest is especially fruitful with wild fungi, and *Tricholoma magnivelare* is the most valuable species

harvested, followed by *Boletus*, *Cantharellus* and *Morchella* (Blatner and Alexander, 1998; Pilz *et al.*, 1999).

There is a long tradition of mushroom use in Mexico, stretching back to pre-Columbian times (Jarvis *et al.*, 2004). Traditional knowledge is best kept in rural, forested areas, which tend to be the poorest (Perez-Moreno *et al.*, 2008). Rural communities traditionally make use of more than 112 species of wild mushrooms (Martinez-Carrera *et al.*, 2002). In the survey of Pellicer-Gonzalez *et al.* (2002), 86.5% of the people living in a community in Puebla consumed wild fungi by gathering, buying or a combination of both. Another study by Montoya *et al.* (2008) revealed that 73.5% of the population of a municipality in the State of Tlaxcala collected wild mushrooms for own consumption and sale. The favourite mushrooms of the Nahua people of Tlaxcala are *Gomphus floccossus*, *Ramaria* spp. and *Boletus pinophilus* (Montoya *et al.*, 2003). Local people of the State of Mexico maintain deep traditional knowledge on mushrooms and consume species that do not have a high commercial value: *Suillus* spp., *Clavulina rugosa*, *Hygrophorus chrysodon* or an unidentified *Lyophyllum* sp. known as "tlalcocomo" (Perez-Moreno *et al.*, 2008). The most valued mushrooms by the highland Maya of Chiapas differ significantly from those traditionally esteemed by Europeans and North Americans: they like *Daldinia* cf. *concentrica*, several *Cortinarius* spp. and *Schizophyllum commune*, and show no interest in boletes and chanterelles (Shepard, Arora and Lampman, 2008).

In general, fungi are not of importance in the culture of Amazonian Indians, either for food or for hallucinogenic purposes (Fidalgo and Prance, 1976; Prance, 1984), but there are some exceptions. The Yanomamo Indians of Brazil do use fungi extensively in their diet. As well as occurring naturally in the area they inhabit, mushrooms are incidentally cultivated by the Indians in the cassava plantations. The debris produced by the slash-and-burn method used in the plantations provides an ideal habitat for wood-rotting mushrooms. Among the species eaten by the Yanomamo are *Favolus brasiliensis*, *Polyporus* spp., *Lentinus* spp., *Pleurotus* spp. and *Collybia* spp. (Fidalgo and Prance, 1976; Prance, 1984). They also eat the sclerotia of *Polyporus indigenus*, known as "Indian bread". Each sclerotium can weigh up to 3 Kg and contains 50% carbohydrates, and the Yanomamo use them as an emergency food because they can be stored for months (Prance, 1984).

Fidalgo and Hirata (1979) reported data from other three Amazonian Indian tribes: the Caiabi Indians use an unidentified Polyporaceae, the Txicão Indians eat *Lentinus crinitus* and *Auricularia fuscosuccinea*, and the Txucarramãe Indians consume *Trametes cubensis*, *Pycnoporus sanguineus*, *Trichaptum trichomallum* and *Auricularia fuscosuccinea*, but only in the case of extreme hunger.

Another example are the Hotï, an indigenous tribe inhabiting the Venezuelan Amazon, who consume around 30 species of wild fungi gathered in high forest habitats, especially *Amauroderma* sp. and *Lenzites acuta* (Zent *et al.*, 2004).

The few Amazonian Indian tribes that use mushrooms happen to be all highly nomadic foraging societies, so this could be an important factor influencing the relative importance of mushrooms in indigenous diet and culture in the Amazonia (Zent, 2008).

The template zones of South America are prolific with fungi, but there are not many records of consumption. In Argentina, *Phlebopus bruchii* is very appreciated due to its resemblance with *Boletus edulis*, and it is consumed fresh since the beginning of the 20th century (Deschamps, 2002). In Uruguay there is an extensive knowledge on wild edible mushrooms mostly due to the European origin of its population. Among the species collected for consumption are *Lactarius deliciosus*, *Suillus granulatus*, *Gymnopilus spectabilis* and *Laetiporus sulphureus* (Deschamps, 2002).

The nutritional value of fungi

Fungi are a good source of digestible proteins, are low in fat and energy, and make a useful contribution to vitamin and mineral intake (De Roman *et al.*, 2006). In general, edible fungi contain 85-95% fresh weight (fw) of water, 16-35% dry weight (dw) of protein, 2-6% dw of fat and 28-39.9% dw of carbohydrates. But the wide variety and abundance of minerals are the most characteristic features of fungi: they are important sources of Cu, Fe, K, Mg, Se, P and Zn in the human diet (Sadler, 2003; Rudawska and Leski, 2004), and they rank among the food stuffs with the lowest Na content, which is of great benefit to the consumer (Seeger *et al.*, 1983; Vetter, 2003). They are rich in unsaturated fatty acids, especially oleic and linoleic acids (Solomko *et al.*, 1984). Judging from their high fibre content and unique fibre composition, fungi have considerable value as source of dietary fibre in human nutrition (Cheung, 1997). A serving (100 g) of mushrooms guarantees from 9 to 40% of the daily recommendation of dietary fibre (Manzi *et al.*, 2001) and 80 g of mushrooms counts as a portion with regard to the advice to eat five portions of fruit and vegetables a day (Sadler, 2003).

Several studies have confirmed that fungi make a useful contribution to vitamin intake, especially vitamins B, D and K, and sometimes vitamins A and C (Sadler, 2003; Sanmee *et al.*, 2003). Mattila *et al.* (1994) showed that *Cantharellus cibarius* and *C. tubaeformis* contained high amounts of ergocalciferol, also known as vitamin D_2, possibly because in the genus *Cantharellus* the pileus and gills are often more effectively exposed to light than they are in other species. For some groups, for example vegetarians or persons allergic to fish, fungi can be thus an important dietary source of vitamin D (Outila *et al.*, 1999).

On the other hand, Degreef *et al.* (1997) agrees that, from the caloric point of view, fungi only play a very limited role (34-131 kcal/100 g), but in some countries the contribution of fungi to the input in minerals, vitamins and essential amino acids in starvation months is vital.

More detailed figures on protein, fat, carbohydrates, fibre, minerals, amino acids, fatty acids and energy content of several species can be found (Adriano and Cruz, 1933; Mukhiibi, 1973; Parent and Thoen, 1977; Alsheikh and Trappe, 1983; Seeger *et al.*, 1983; Coli *et al.*, 1988; Alofe, 1991; Botha and Eicker, 1992; Bokhary and Parvez, 1993; Mattila *et al.*, 1994; Vetter, 1994; Eisenhut *et al.*, 1995; Alofe *et al.*, 1996; Degreef *et al.*, 1997; Leon-Guzman *et al.*, 1997; Longvah and Deosthale, 1998; Outila *et al.*, 1999; Vetter, 1999; Aaronson, 2000; Ilievska and Petrovska, 2000; Diez and Alvarez, 2001; Manzi *et al.*, 2001; Caglarirmak *et al.*, 2002; Sadler 2003; Sanmee *et al.*, 2003; Didukh *et al.*, 2004; Agrahar-Murugkar and Subbulakshmi, 2005; Vetter, 2005; Yildiz *et al.*, 2005).

The contribution of wild fungi to income

There has always been an extensive and regular trade in wild fungi but, until the last 15 – 20 years, little has been known about collectors and traders (Boa, 2004). Wild fungi are today an important source of income in both developing and developed countries (Hosford *et al.*, 1997; Wong *et al.*, 2001; Boa, 2004). Detailed figures are hard to obtain, and fungi prices exhibit a high degree of daily and year-to-year variation. They are strongly influenced by weather conditions and by the delivered prices of competing suppliers around the world (Blatner and Alexander, 1998). However, the studies published so far bring clear evidence of the economic significance of wild fungi, especially for the stakeholders at the base of the market chain (Table 12.1).

Table 12.1. Average prices paid to harvesters of wild fungi as recorded in the literature.

Species	Country	Original price per kg[1]	Price per kg converted into USD[2]	Reference
Amanita caesarea	Nepal	60-90 rupees	1.5-2.25	Adhikari and Durrieu (1996)
	Mexico	2 USD	2	Martinez-Carrera *et al.* (2005)
	Mexico	2.7-4.5 USD	2.7-4.5	Montoya *et al.* (2008)
Amanita zambiana	Zimbabwe	3 USD	3	Masuka (2002)
Boletus edulis	Mexico	1.5 USD	1.5	Martinez-Carrera *et al.* (2005)
	Spain	2-8 €	2.9-11.7	Bellon *et al.* (2004)
Boletus cf. *pinophilus*	Mexico	1-3.6 USD	1-3.6	Montoya *et al.* (2008)
Boletus spp.	Finland	3 USD	3	Härkönen (1998)
	Spain	8 €	11.7	Hermida (2004)
	USA	12 USD	12	Blatner & Alexander(1998)
Cantharellus cibarius	Spain	10-20 €	14.7-29.4	Bellon *et al.* (2004)
	Spain	10.50 €	15.4	Hermida (2004)
	Nepal	0.7-1.5 USD	0.7-1.5	Christensen *et al.* (2008)

Cantharellus lutescens	Spain	5.90 €	8.7	Hermida (2004)
Cantharellus miniatescens	Zimbabwe	2 USD	2	Masuka (2002)
Cantharellus spp.	Finland	8 USD	8	Härkönen (1998)
	USA	6.6 USD	6.6	Blatner and Alexander (1998)
Cantharellus tubaeformis	Spain	5.90 €	8.7	Hermida (2004)
Collybia dryophila	Mexico	3 USD	3	Martinez-Carrera *et al.* (2005)
Craterellus cornucopioides	Spain	5.90 €	8.7	Hermida (2004)
Entoloma cf. *clypeatum*	Mexico	1.36 USD	1.36	Montoya *et al.* (2008)
Gomphus floccosus	Mexico	0.9-1.36 USD	0.9-1.36	Montoya *et al.* (2008)
Grifola frondosa	Nepal	30-40 rupees	0.75-1	Adhikari and Durrieu (1996)
	Nepal	1.4-2.1 USD	1.4-2.1	Christensen *et al.* (2008)
Gymnopilus spectabilis	Uruguay	2 USD	2	Deschamps (2002)
Helvella spp.	Mexico	3 USD	3	Martinez-Carrera *et al.* (2005)
Hydnum repandum	USA	7.2 USD	7.2	Blatner and Alexander (1998)
Hydnum spp.	Spain	2.80 €	4.1	Hermida (2004)
Laccaria spp.	Nepal	0.4-1.8 USD	0.4-1.8	Christensen *et al.* (2008)
Lactarius deliciosus	Argentina	1 USD	1	Deschamps (2002)
	Chile	0.5 USD	0.5	Deschamps (2002)
	Spain	3-10 €	4.4-14.7	Bellon *et al.* (2004)
	Spain	7 €	10.3	Hermida (2004)
	Spain	2 €	2.9	De Roman and Boa (2006)
	Uruguay	2 USD	2	Deschamps (2002)
Lactarius kabansus	Zimbabwe	1 USD	1	Masuka (2002)
Lactarius thakalorum	Nepal	0.7-2.1 USD	0.7-2.1	Christensen *et al.* (2008)
Lactarius spp.	Finland	2 USD	2	Härkönen (1998)
Laetiporus sulphureus	Nepal	30-40 rupees	0.75-1	Adhikari and Durrieu (1996)
	Nepal	0.6-8.1 USD	0.6-8.1	Christensen *et al.* (2008)
	Uruguay	2 USD	2	Deschamps (2002)
Lyophyllum decastes	Mexico	0.9-1.63 USD	0.9-1.63	Montoya *et al.* (2008)
Lyophyllum sp.	Mexico	1.36-4.5 USD	1.36-4.5	Montoya *et al.* (2008)
Morchella conica	Nepal	2.9-11.4 USD	2.9-11.4	Christensen *et al.* (2008)
Morchella esculenta	Mexico	5.2 USD	5.2	Martinez-Carrera *et al.* (2005)
Morchella spp.	Canada	7 USD	7	Wurtz *et al.* (2005)
	Nepal	3000-4000 rupees DW[3]	75-100 DW	Adhikari and Durrieu (1996)

	USA	10 USD	10	Blatner and Alexander (1998)
	USA	8-12 USD	8-12	Wurtz *et al.* (2005)
Phlebopus bruchii	Argentina	20 USD DW	20 DW	Deschamps (2002)
Pleurotus ostreatus	Nepal	30-40 rupees	0.75-1	Adhikari and Durrieu (1996)
Ramaria spp.	Nepal	0.6-1.9 USD	0.6-1.9	Christensen *et al.* (2008)
	Mexico	0.9-1.4 USD	0.9-1.4	Montoya *et al.* (2008)
Russula delica	Nepal	0.7-1.5 USD	0.7-1.5	Christensen *et al.* (2008)
Sparassis crispa	USA	6 USD	6	Blatner and Alexander (1998)
Suillus granulatus	Uruguay	2 USD	2	Deschamps (2002)
Suillus luteus	Chile	0.5 USD	0.5	Deschamps (2002)
Termitomyces heimii	India	6-12 rupees	0.15-0.3	Harsh *et al.* (1999)
	Nepal	2.1-2.9 USD	2.1-2.9	Christensen *et al.* (2008)
Tricholoma magnivelare	Mexico	32-52 USD	32-52	Martinez-Carrera *et al.* (2002)
	USA	41 USD	41	Blatner and Alexander (1998)
Tricholoma terreum	Nepal	0.7-1.5 USD	0.7-1.5	Christensen *et al.* (2008)
Tuber spp.	USA	54.6 USD	54.6	Blatner and Alexander (1998)
Xerocomus badius	Spain	0.90 €	1.3	Hermida (2004)

[1] Kg of fresh weight unless otherwise stated. [2] Exchange rates as of December 2007.
[3] DW = dry weight

There are surprisingly few accounts of the trade in wild fungi in Europe. Notable exceptions are the studies by Dyke and Newton (1999) in Scotland and De Roman and Boa (2006) in Spain. Dyke and Newton (1999) found that the total wild fungi harvest in Scotland is worth some £406,000 per year. Harvesters benefit from casual earnings averaging £28.70 per week, which represents 6.6% of their annual income. In a rural community in northern Spain, a family of four could make a profit of 5600-8400 € in a season collecting *Lactarius deliciosus* (De Roman and Boa, 2006). The average annual income per family in the area is 18727 €, so it is obvious that fungi picking is a remarkable means of obtaining an additional income. It is also worth to note that the black truffle, *Tuber melanosporum*, is exported from France, Italy and Spain, but detailed figures on the income produced are usually hard to obtain due to the secrecy with which the trade takes place.

In Africa there are only records from Zimbabwe, where pickers get on average USD 90-130 per month during the period December-March. The contribution of fungi to the household income is more than agricultural worker's income during this period (Masuka, 2002). And in Malawi, Boa (2002) noticed that fungi collection and trade had increased significantly over the past decade, driven by the desperate poverty of the rural poor, who are attracted to fungi collection and sale because it does not require an initial investment of land or money.

In Arab countries, *Tirmania* is marketed at prices comparable to meat and indeed used instead of it (Alsheikh and Trappe 1983). In Bahrain, desert truffles (*Tirmania nivea* and *Terferzia claveryi*) are collected as a means for financial supplement (Mandeel and Al-Laith, 2007).

In the Indian state of Madhya Pradesh, the sale of edible fungi contributes about 2% to the annual income of a tribal family (Harsh *et al.*, 1999), whereas in the Great Himalayan National Park in Himachal Pradesh the harvest of *Morchella esculenta* contributes on average 20-30% of the annual income per household (Singh and Rawat, 2000). In Nepal, the most dedicated collectors earn between NPR 2,000 and NPR 12,000 per year from selling mushrooms, i.e., between USD 28 and USD 171, which equals one to six months of unskilled labour salary (Christensen *et al.*, 2008).

In China's northwest Yunnan province 50-80% of household income is generated by the harvest and sale of matsutake (Yang *et al.*, 2008). The collection of *Cordyceps sinensis* is the most important source of cash for rural households in contemporary Tibet, accounting for 70-90% of a family's annual cash income and contributing at least USD 225 million to the Tibet Autonomous Region's GDP (Winkler, 2008).

In the Pacific Northwest region of the USA the wild mushroom industry was estimated at USD 41.1 million in 1992 (Pilz *et al.*, 1999) and 35% of pickers use fungus harvest as their main source of income (Dyke and Newton, 1999). Overexploitation of the natural resources is feared and different regulatory systems have been developed, for example in Oregon (McLain, 2008) and California (Arora, 2008). *Tricholoma magnivelare* is the most valuable mushroom collected. During the height of the season harvesters can earn between USD 200-500 a day (Rowe, 1997).

In Mexico, the harvest and sale of wild fungi represent between 8.9% and 19.2% from overall incomes in rural communities (Martinez-Carrera *et al.*, 2002; Pellicer-Gonzalez *et al.*, 2002). The most important mushroom collected is *Tricholoma magnivelare*. It is commonly known as "hongo blanco" and mainly exported to Japan. It is gathered commercially by peasants since the middle of the 1980's mainly in the States of Durango, Hidalgo, Mexico, Michoacan, Oaxaca, Puebla and Veracruz. But not only "hongo blanco" is collected to be sold. Many species can be found in rural markets, such as in the State of Tlaxcala, where *Boletus pinophilus*, *Amanita caesarea* and *Agaricus campestris* show the highest prices and demand by consumers (Montoya *et al.*, 2001).

A study undertaken in a municipality in the VII region of Chile revealed that those families who collect wild fungi have a monthly average income of 70,000 Chilean pesos, well above the 30,000 Chilean pesos earned by families not involved in the harvest of wild fungi. Some pickers are even reported to earn 500,000 Chilean pesos per season (FAO, 1993).

Market demand for wild mushrooms and other non-wood forest products has increased to the extent that their commercial value may surpass the value of

timber-based trade (Arnolds, 1995). In France, a productivity of 10-15 kg/Ha of *Boletus edulis* equals the mean revenue obtained with the wood industry (Parant, 1991). In Spain, if the wood industry is below $2m^3$/Ha and year, which applies to most areas of the country, then the revenue obtained with the collection of fungi is greater than that obtained with the wood (Oria de Rueda, 1989).

But not only the harvest and sale of wild fungi are of economic importance, mycotourism is also a source of income in some countries, for example Spain (Bellon *et al.*, 2004). And in some European countries, such as Finland, the integration of wild fungi harvesting into wider cultural life is more important than raw economic value (Dyke, 2003).

The medicinal value of fungi

Records of fungi being used in folk medicine in the Orient go back to the 1st century B.C. in China (Ying *et al.*, 1987). From the Western perspective though, it is often difficult to evaluate the effectiveness of fungi as medicines due to the discrepancy between Eastern and Western mind-body concepts (Birks, 1991).

Ethnomycological research has revealed the current use of fungi as medicines in many societies around the world. The traditional use of puffballs (Lycoperdales and Tulostomatales) for medicinal purposes is universal (Birks, 1991). To cite just a few examples of this ethnomycological use of medicinal mushrooms, Harsh *et al.* (1999) and Rai *et al.* (1993) found wide ethnomycological knowledge and use of medicinal fungi among tribes from Madhya Pradesh, in India. Among the species used were *Agaricus* sp. for goitres, *Astraeus hygrometricus* for burns, *Bovista pusilla* to staunch bleeding, *Calvatia cyathiformis* for healing wounds, *Cyathus sterocoreus* and *C. limbatus* for sore eyes, *Ganoderma lucidum* for cataract and asthma, *Micropus xanthopus* for fever and earache, *Phallus impudicus* and P. *rubicundus* for typhoid and labour pain, *Termitomyces microcarpus* for partial paralysis and as a tonic for weakness, and *Xylaria polymorpha* to induce lactation. In Nepal, Adhikari and Durrieu (1996) recorded the use of spores of *Lycoperdon* for cicatrisation of injuries, and of *Coriolus hirsutus*, *Fistulina hepatica*, *Ganoderma lucidum* and *Inonotus hispidus* to heal cuts and burns. The Nahua people of Tlaxcala, in Mexico, make a broad use of *Ustilago maydis*: to heal wounds, dry the navels of newborns, stop hemorrhages, heal animal bites and to alleviate dehydration produced by consumption of too much alcohol (Montoya *et al.*, 2003).

On the other hand, scientists have undertaken many studies under *in vitro* and *in vivo* conditions to demonstrate the effectiveness of these traditional remedies and explain their mechanisms of action. Biologically active substances (polysaccharides, dietary fibers, lectins and terpenoids) of higher Basidiomycetes mushrooms show immunomodulating, antitumor, antioxidant, antidiabetic, hypocholesterolemic, antibiotic, antimicrobial and antiparasitic effects (Wasser and Weiss, 1999). The best-known species and thus the most commonly used in medicine nowadays are *Claviceps purpurea*, *Cordyceps sinensis*, *Ganoderma*

lucidum, *Laricifomes officinalis*, *Lentinula edodes* and *Trametes versicolor* (Molitoris, 2002).

Didukh *et al.* (2004) provide a thorough review on the medicinal properties of Agaricaceae species, with focus on *Agaricus brasiliensis*. The medicinal properties that have been confirmed for these fungi so far are antitumor, antiviral, antigenotoxic and antimutagenic, antioxidant and immunomodulatory activities. Takaku *et al.* (2001) report that *Agaricus brasiliensis* is used by 300,000-500,000 people worldwide for the prevention of cancer and/or as an adjuvant with cancer chemotherapy drugs after the removal of a malignant tumor.

Lakshmi *et al.* (2004) proved the antioxidant activity of *Phellinus rimosus*, *Pleurotus florida*, *Pleurotus sajor-caju*, and *Ganoderma lucidum*. Cellular damage caused by reactive oxygen species has been implicated in several diseases, and hence antioxidants have significant importance in human health.

Ling *et al.* (1990) report that *Collybia velutipes* is a protective factor against increased serum cholesterol level and promotes the formation of haemoglobin in rats. Ooi (2001) has focused on the effects of selected Chinese medicinal fungi, namely the immunomodulation and antitumor activity of *Tricholoma giganteum*, the blood pressure lowering action and mechanism of *Volvariella volvacea* and the liver protective effect of *Trametes versicolor*.

In a pilot clinical test in Africa, a dietary supplement of *Ganoderma lucidum* strengthened the health of HIV/AIDS patients by repairing the damaged lining of the intestine and improving food absorption (Mshigeni *et al.*, 2005).

About 650 species of higher Basidiomycetes have been found to exhibit antitumor activity, mostly by activation of the immune response of the host organism, also known as immunomodulation (Wasser, 2002). At the moment there are 5 mushroom preparations, all β-D-glucans, i.e., high-molecular-weight polysaccharides, that have shown clinically significant effects against human cancers: Lentinan from *Lentinula edodes*, D-fraction from *Grifola frondosa*, schizophyllan from *Schizophyllum commune* and PSK and PSP from *Trametes versicolor* (Zaidman *et al.*, 2005). Ikekawa (2001) also reports the high antitumor activity and the preventive effect in tumor metastasis that *Flammulina velutipes* and *Hypsizygus marmoreus* have.

The trend for future research is to focus on low-molecular-weight compounds, among which many substances of therapeutic interest have been newly discovered, but they still need to go through advanced phases of clinical evaluation.

Concluding remarks

Fungi have a balanced and high nutritional value, are rich sources of protein and fibre, low in fat, and make a useful contribution to mineral and vitamin intake. They are promising alternative sources of food in a world where food shortage due to the rapid increase in human populations is becoming a serious

problem (Ghosh, 2004). In developing countries, fungi have a high nutritional significance at a very crucial time of the year, the traditional hunger months at the beginning of the rains (Morris, 1984; Degreef *et al.*, 1997). And in developed countries, the regular use of wild fungi as food is considered to provide a healthy and balanced diet, what is called nowadays "functional foods", i.e. materials which are beneficial but not absolutely vital for our bodies (Tudge, 2001; Härkönen, 2002).

The demand for wild fungi grows from year to year, and all stakeholders benefit from the increase in global trade. Wild fungi are an important source of additional income, especially in less favoured rural areas. More research needs to be done in order to get a deeper insight into the market chain and to get more accurate information on the wider social, economic and environmental issues that concern this activity. As trade increases, wild fungi should be taken into account in forest management programmes in order to guarantee their sustainable development.

Fungi are also expected to have an increasing importance in medicine and biotechnology in the future due to their unique biosynthetic capabilities and metabolic products. A broader spectrum of species needs to be screened for biologically active substances, and more clinical studies are needed in order to prove their medicinal value and understand their mechanisms of action.

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CHAPTER - 13

MERCURY IN CERTAIN MUSHROOM SPECIES IN POLAND

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Introduction

Many wild-grown higher fungi (mushrooms, macromycetes) often are able to pick-up from soil substrate uncontaminated anthropogenically and bioconcentrate in their fruit bodies various metallic elements and/or metalloids to strikingly great concentration. This feature is known among mycorrhizal fungi and humus decomposers. Even, some species are able to hyperaccumulate certain elements. The examples of hyperacumulating species are: Fly Agaric (*Amanita muscaria*) rich in vanadium found in organovanadium compound amavadine (mean up to 300 μg V/g dry weight); the Amethyst Deceiver (*Laccaria amethistina*) and Common Deceiver (*Laccaria laccata*) rich in arsenic found in arsenobetaine and some other arsenocompounds (0.43 and 250 μg As/g dw), and also Common Earth-fan (*Thelephora terresris*) with the total As at 37-39 μg/g dw; the Goat's Foot (*Albatrellus pes-caprae*) rich in selenium found in several seleno compounds concentration up to 370 μg/g dw; the Variegated Bolete (*Suillus granulatus*), which is rich in iron (mean up to 3600±510 μg/g dw) or found recently to be particularly rich in silver the Amanitas (*Amanita strobiliformis* and *A. solitaria*) with 200-1300 μg Ag/g dw); while Bay Bolete (*Xerocomus badius*) is abundant in radiocesium (Kneifel and Bayer, 1973; Stijve, 1977; Koch *et al.*, 1987; Stijve and Bourqui, 1991; Mietelski *et al.*, 1994; Slekovec and Irgolic, 1996; Stijve *et al.*, 1998; Falandysz *et al.*, 2001b; Isiloglu *et al.*, 2001; Borovička *et al.*, 2007).

Therefore, numerous edible wild grown mushroom species are usually rich in some mineral constituents. Elemets such as selenium, copper or zinc in

mushroom could be vital to human and animal health but certain are problematic, and example is cadmium, lead or mercury (Stijve and Besson, 1976; Varo *et al.*, 1980; Bargagli and Baldi, 1984; Kalac *et al.*, 1991; Falandysz, 2006, 2008; Garcia *et al.*, 2009).

The genetic and ecological factors (age of mycelium, production of chelating agents, mobilization and immobilization of metals, yield of fruit bodies, soil substrate characteristic), in addition to certain poorly known factors (abundance and bioavability of elements and impact of their oxidation state or chemical form in substrate on bioconcentration potential or exclusion, possibility of biomethylation of some elements by mushroom and enhanced accumulation of methylated species in the flesh; abundance of possible ligands in fruit body including sulfhydryl groups, disulfides or methionine; passive or active uptake, and for many species also impact of mycorrhiza), determine the ability of mycelium (hyphae) to absorb, transform, translocate, excrete and accumulate many elements at relatively great concentrations in the fruit bodies of fungi (Seeger, 1976; Quinche, 1976; Aichberger, 1977; Lodenius *et al.*, 1981; Zurera *et al.*, 1986; Falandysz and Szajek, 1994; Falandysz *et al.*, 1995, 1997; Falandysz and Kryszewski, 1996; Kalač and Slapetova, 1997; Vetter and Berta 1997; Gast *et al.*, 1988; Gadd, 2007).

These relationships between abundance and bioavailability of elements from soil or other substrate to the fungal mycelium and their further transport to the fruit body and accumulation in its various constituent parts seem to be very complex (Gadd, 2007). Additionally, it become known that for some mushroom species and elements the ability of mycelium to colonize various substrates and to actively or passively absorb and further accumulate elements in the fruit bodies is highly species-specific. This species-specific oriented accumulation of certain metallic elements, metalloids or methylmercury by certain fungi doesn't seems to exclude possibility of theirs simultaneous accumulation in a substrate-dependent, to some degree, concentration mode as observed at several highly contaminated sites due to pollution because of the lead, mercury or other metal-ore smelters, etc. (Kalac *et al.*, 1991 ,1996; Fischer *et al.*, 1995; Barcan *et al.*, 1998). Nevertheless, it does not seem to be realistic a suspicion that any higher fungi could be like a passive accumulator (or sampler) of any environmentally relevant metallic element or metalloid diffused in the substrate.

Many fungi species accumulate mercury in the fruit bodies. This feature is found both between these of saprophytic and mycorrhizal nature, and including edible species. High accumulation of mercury by mushrooms is a feature rather rare when compared to vascular plants. In the case of fungi growing in field conditions, the relationships between mercury content of the fruit body and soil or other substrate, if any, are frequently unclear. Moreover, to have a better insight, at least, instead of "the total" mercury all its possible forms (speciation) have to be determined both in substrate and fungi. Quality of the substrate, as related to the level and chemical form of metallic element or metalloid present, is a feature which can highly impact element's content of the fungi's fruit body, as

observed both under artificial laboratory or field condition and in nature (Rauter, 1975; Bressa *et al.*, 1988; Turnau and Kozłowska, 1991; Falandysz *et al.*, 1994; Fischer *et al.*, 1995; Müller *et al.*, 1997). At the mercury-contaminated sites (mines, smelters, chlor-alkali plants) hyperaccumulation of this element by mushrooms is a well-known fact (Rauter, 1975; Lodenius and Herranen, 1981; Bargagli and Baldi, 1984; Kalač *et al.*, 1991 and 1996; Fischer *et al.*, 1995; Svoboda *et al.*, 2000). Some wild growing mushroom species efficiently bioconcentrate mercury even or especially when the substrate contamination is very low. They could contain mercury in fruit body at surprisingly great concentration when compared to geochemical soil background (Fischer *et al.*, 1995; Falandysz and Chwir, 1997; Alonso *et al.*, 2000; Falandysz *et al.*, 2001a-b and 2003a; Falandysz, 2002; Tuzen and Soylak, 2005; Cocchi *et al.*, 2006).

Elemental mercury due its physicochemical properties resulting in easy evaporation, high atmospheric residence time and further long-range atmospheric transportation is unique amongst metallic elements. Mercury could be easily released to the global environment due to thermal processes related to fossil fuels combustion, waste incineration, fires etc., and is one of definitely toxic elements to human and some animal life, and especially in form of environmentally highly relevant methylmercury. Further, possibility of mercury biomethylation to highly neurotoxic monomethylmercury ion makes this element one of the most problematic environmental and food contaminant of highest concern. Methylmercury forms only a small portion of the total mercury found usually in the fruit bodies of higher mushrooms. Nevertheless, methylmercury can be a much more efficiently bioconcentrated by some species than inorganic mercury compounds, and even some mushroom species may be could be able to methylate mercury (Fischer *et al.*, 1995).

There is some evidence also that mercury in some species of wild mushrooms at the unpolluted regions could be associated with selenium and both elements could be to some degree species-dependent accumulated (Stijve and Cardinale, 1974; Falandysz, 2006). Neverthless, there is scarcity of data on mercury and selenium relationships as related to higher mushrooms both at the uncontaminated and contaminated with mercury areas.

A phenomenon and mechanisms of elements accumulation by fungi and theirs possible bioindicative potential as well as mineral element content, speciation and nutritional status of wild grown edible but also cultivated species remain among topics for elucidation. Within this chapter are discussed problems of mercury bioconcentration potential by and its content of surface soil layer underneath to the fruit bodies of six fungi species from the stands accross Poland and some other countries (Tables 13.1-13.7). These species are edible King Bolete (*Boletus edulis*), Bay Bolete (*Xerocomus badius*), Brown Birch Scaber Stalk (*Leccinum scabrum*), Parasol Mushroom (*Macrolepiota procera*) and inedible Fly Agaric (*Amanita muscaria*) and Poison Pax (*Paxillus involutus*).

Soil/substrate Hg content

The underlying to six fungi species mentioned surface soil layer (0-10 or 0-15 cm) exhibited usually low or very low mercury contamination (Table 13.1). Taking into account data for numerous sites surveyed (Table 13.2), because of low mercury content of surface soil horizon a lack of any point, local or regional important sources of pollution with this element in Poland seems evident. Mercury at slightly elevated concentrations could be noted at few of the sites surveyed, and its occurrence there could be related to impact of large cities or a direct human industrially like activity. Hard coal and lignite combustion for electric energy and heat production probably is a main source of mercury diffusion to ambient air in Poland.

In details, total variation of mercury concentrations in soil substrate for King Bolete, Bay Bolete, Brown Birch Scaber Stalk, Parasol Mushroom, Fly Agaric and Poison Pax from various sites across Poland was, respectively, between 0.01 and 0.08, 0.0041-0.35, 0.011-0.10, 0.013-0.54, 0.002-0.49 and 0.01-0.14 μg/g dw (Table 13.1).

As mentioned earlier, in certain species and stands mercury content of surface soil was somehow elevated, when compared to most of the sites surveyed. Gostyńsko-Włocławskie Forest in the County of Starachowice and the Lubelska Upland, where mercury content in soil was from 0.045±0.016 to 0.066±0.018 μg/g dw could be axample. Mercury content of soil was greater ($p < 0.05$; U Man Whitney test) there when compared to other sites for Brown Birch Scaber Stalk, and including a southernmoust the Kłodzka Valley site in the Sudety Mountains (Falandysz and Bielawski, 2007).

Table 13.1. Summary data on mercury content of soil underneath to the fruit bodies of six fungi species from the spatially distant sites in Poland (μg/g dw) (Falandysz and Bielawski, 2007; Falandysz and Brzostowski, 2007; Falandysz *et al.*, 2007a,b,f; Falandysz and Gucia, 2008)

Soil underlying to the species	Means and SD	Medians	Total range
King Bolete (13 sites)	0.02±0.01 - 0.05 ± 0.02	0.02-0.05	0.01-0.08
Bay Bolete (13 sites)	0.0074±0.0033 - 0.21±0.08	0.0070-0.21	0.0041-0.35
Brown Birch Scaber Stalk* (12 sites)	0.026±0.010 - 0.066±0.018	0.023-0.032	0.011-0.10
Parasol Mushroom (19 sites)	0.022±0.011 - 0.36 ± 0.16	0.022-0.40	0.013-0.54
Fly Agaric (14 sites)	0.011±0.006 - 0.19 ± 0.12	0.010-0.14	0.002-0.49
Poison Pax (14 sites)	0.02±0.01 - 0.10 ± 0.04	0.01-0.10	0.01-0.14

*Aqua regia extraction (otherwise hot extraction with concentrated nitric acid)

In the case of Fly Agaric, total mercury content of the top layer of forest soil in all cases was < 0.5 μg/g dw (Table 13.1). The value of arithmetic mean of mercury concentration for 70-pooled soil samples for this species was 0.055±0.081 (0.002-0.49) μg/g dw, and median was 0.025 μg/g dw. Nevertheless, some variations of soil mercury content between the sites surveyed were

statistically significant (<0.05 p <0.01; U Mann-Whitney test). The total mercury content of soil from the Starachowickie forest was again (as in the case of Brown Bich Scaber Stalk), relatively greater, when compared to other sites where fruit bodies of Fly Agaric were collected (Table 13.1) (Falandysz *et al.*, 2007f).

For Poison Pax at two sites in Tucholskie Forest mercury content of soil was from 0.06±0.02 to 0.07±0.04 μg/g dw, and 0.10±0.04 and 0.09±0.04 μg/g dw were both for area close to the industrialized town of the Starachowice and Kłodzka Valley in the Sudety Mountains, respectively (Table 13.7). A similar pattern of mercury concentrations in forest soil accross Poland as found for six fungi species mentioned was observed in a parallel studies of some other mushrooms (Falandysz and Chwir, 1997; Falandysz *et al.*, 2002a).

A mean value of total mercury content for European arable top soil layer is close to 0.1 μg/g dw, and for the forested top soil usually is between 0.05 and 0.15 μg/g dw (Rundgren *et al.*, 1992; Falandysz *et al.*, 2002a; Falandysz *et al.*, 2007a). A value of around 0.7 μg Hg/g dw was suggested as probable natural concentration in the humus layer in Sweden (Swedish EPA, 1991). No one of soil samples underneath to the fruit bodies of these six fungi species (Table 13.1) exceeded value of 0.7 μg Hg/g dw.

The mushrooms caps, stipes and the whole fruit bodies Hg content

Natural mercury content of fruit bodies of King Bolete and Parasol Mushroom collected from an unpolluted with this element sites is relatively high and, when compared to both species a much weaker accumulators are Bay Bolete, Brown Birch Scaber Stalk, Fly Agaric or Pioson Pax. Even these species considered as weaker mercury accumulators when grown up at contaminated sites could contain this element in fruit body at highly elevated concentration (Table 13.2-13.7). The means of total mercury content of King Bolete for numerous sites in Poland ranged from 1.2±1.4 to 7.6±3.1 μg/g dw in caps (total range from 0.02 to 14 μg/g dw), and from 0.84±0.74 to 3.8±1.8 μg/g dw in stipes (total range from 0.03 to 6.7 μg/g dw). The King Bolete specimens with elevated mercury content were collected at the Świętokrzyski Landscape Park. This site is localized in southern-central part of Poland, and which region seems to be impacted by industrial activities much harder, when compared to the northern part of the country. Also King Bolete collected from the Płocka Hollow area in central part of Poland, when compared to specimens from some other sites showed relatively elevated mercury content ($p < 0.01$; U Mann-Whitney test) (Table 13.2).

The total mercury at relatively elevated concentration was also in King Bolete from some sites considered as more or less unpolluted with this element in Europe (Table 13.2). In a study in Czech Republic the specimens of King Bolete collected from the Precambrian shales area contained 7.9±0.3 μg Hg/g dw, while much less noted for the Paleozoic graywache area (Table 13.2). The fruit bodies of King Bolete at polluted with mercury site can contain up to 63 μg Hg/g dw,

and this value apparently highly exceeds what could be found in this species grown-up in uncontaminated sites.

Table 13.2. Review of data on concentration, cap to stipe concentration quotient ($Q_{C/S}$) and bioconcentration factor (BCF) values of mercury in King Bolete (*Boletus edulis*) in Poland and other countries (μg/g dw; arithmetic mean, SD, range and median value, respectively)

Country, site and year of collection	n	Fruit body		$Q_{C/S}$	BCF	Reference
		Whole	Cap			
Uncontaminated sites						
Norway, p. 1978	1		0.89	2.8		Allen and Steinnes, 1978
Finland, 1976	2	0.094 (0.066-0.12)				Varo *et al.*, 1980
Finland, rural site, 1979	7	0.7 (0.01-1.5)				Kuusi *et al.*, 1981
Finland, urban site, 1979	6	4.1 (0.4-19)				Kuusi *et al.*, 1981
Finland, Helsinki, 1979-84	2	2.9 (2.5-3.2)				Kojo and Lodenius, 1989
Finland, Vousaari, 1978	1	1.5				Laaksovirta and Lodenius, 1979
Finland, 1979	4	0.61±0.15 (0.41-0.73)				Lodenius and Herranen, 1981
Finland, Tikkurila, lead processing area, 1980-81	5	0.9 (0.3-1.5)				Liukkonen-Lilja *et al.* 1981
Finland, central and southern, 1976-1999	21	3.3				Alfthan, 2000
Sweden, Vasterbotten, Umeå, 1995	16		1.2±1.4 (0.06-5.4)	2.6±1.0		Falandysz *et al.*, 2001a
Denmark, 1980-81		1.6				Andersen *et al.* 1982
Poland, Gubin, 1994	16		3.0±1.2 (1.2-4.5)	2.0		Falandysz and Kryszewski, 1996
Poland, Pomorskie Voivodeship, Buszkowy, 1994	4		5.5±1.9 (3.9-8.2)			Falandysz *et al.* 2001b
Poland, Tarnobrzeska Plain, 1995	15		3.4±1.4 (1.3-5.6)	1.6±0.4		Falandysz, 2002
Poland, Trójmiejski Landscape Park, 1996	15		2.9±1.4 (1.2-6.9)	2.6±1.1		Falandysz *et al.*, 2003a
Poland, Wdzydze Landscape Park, 1995-96	15		2.6±2.0 (0.9-7.1)	1.6±1.7		Falandysz *et al.*, 2003b

Poland, Wdzydze Landscape Park, 1996-97	15	0.39±0.40 (0.13-1.8)	2.5±0.7		Falandysz *et al.*, 2003e
Poland, Warmia & Mazury Voivodeship 1997-98	16	3.0±1.6 (1.8-7.1)	1.9±0.8		Falandysz *et al.*, 2003d
Poland, Zaborski Landscape Park, 1997-98	15	2.6±1.2 (0.8-5.9) 1.5	2.3±0.8	70±68	Falandysz *et al.*, 2002d
Poland, Augustowska Forest, 1997-98	16	2.3±1.1 (0.6-4.5)	2.2±0.4		Falandysz *et al.*, 2002a
Poland, Kaszuby, 1998	15	2.3±0.9 (0.97-3.7)			Falandysz *et al.*, 2008a
Poland, Borecka Forest, 1998	+16(20)	9.9±2.7 (4.0-14)	2.0±0.6		Falandysz *et al.*, 2002c
Poland, Borecka Forest, 1998	7	2.0±1.6 (0.8-3.1)			Falandysz *et al.*, 2008a
Poland, Warmia & Mazury Voivodeship, Szczybay. Oromskie., 1998	16 (27)	3.6±1.4 (1.8-6.4)	2.4±1.1		Falandysz *et al.*, 2002c
Poland, Augustowska Forest, 1999	16	2.6±0.5 (1.9-3.5)	2.4±0.9	76±22	Falandysz *et al.*, 2007a
Poland, Tatra Mount., Chochołowska Dale, 1999	11	0.95±0.63 (0.14-2.2)			Falandysz *et al.*, 2008a
Poland, Tatra Mount., Chochołowska Dale, 1999	14	2.3±1.2 (0.67-4.3)	3.2±2.1	53±22	Falandysz *et al.*, 2007a
Poland, Sudety Mount., Kłodzka Valley, 2000	10	2.0±0.7 (1.3-3.1) 2.2	1.1±0.1	41±6	Falandysz *et al.*, 2007a
Poland, Sudety Mount., Kłodzka Valley, 2000	10	2.0±0.3 (1.0-2.8)			Falandysz *et al.*, 2008a
Poland, Kaszuby, 2000	15	2.8±1.0 (1.6-4.5) 2.5	1.9±0.4	81±10	Falandysz *et al.*, 2007a
Poland, Wejherowo Forest, 2000	5	2.6±1.2 (1.3-5.6) 23	2.6±1.5	99±31	Falandysz *et al.*, 2007a
Poland, Sobieszewska Island, Gdańsk, 2000	9	3.1±0.7 (2.1-4.2) 2.8	1.9±0.5	130±39	Falandysz *et al.*, 2007a
Poland, Olsztyn Lakes region, 2000	14	1.2±1.4 (0.02-4.0) 0.73	1.4	53±12	Falandysz *et al.*, 2007a
Poland, County of Kętrzyn, 2000	15	2.2±0.7 (1.1-3.9) 2.1	1.9±0.8	73±17	Falandysz *et al.*, 2007a

Poland, Giżycko Forest, 2000	15		3.3±2.1 (0.57-8.6) 3.0	2.3±1.7	100±13	Falandysz *et al.*, 2007a
Poland, Bydgoszcz Forest, 2000	15		1.4±0.4 (0.70-2.3) 1.4	1.4±0.7	57±10	Falandysz *et al.*, 2007a
Poland, Płocka Valley, 2000	15		4.9±1.4 (2.1-7.9) 5.1	2.3±0.7	110±24	Falandysz *et al.*, 2007a
Poland, Świętokrzyski National Park, 2000	15		7.6±3.1 (4.0-14) 6.3	2.0±0.4	110±40	Falandysz *et al.*, 2007a
Poland, Tatra Mount., Chochołowska Dale, 2000	14		2.3±1.2 (0.67-4.3) 2.1	3.2±2.1	53±26	Falandysz *et al.*, 2007a
Poland, Tucholskie Forest, 2001	15		3.2±1.2 (0.5-5.5) 3.0	2.0±0.3	63±13	Falandysz *et al.*, 2007a
Slovenia, 1972	3	3.5 (2.4-4.4)[c]				Stegnar *et al.*, 1973
Slovenia, Dvor, 1978	1	0.89				Byrne *et al.*, 1979
Bohemia, 1986-87	19	2.3±0.9 (0.95-4.4)				Kalač *et al.*, 1989 and 1991
Bohemia, southern part, 1994-96	8	4.6±2.5 (0.73-7.7)				Kalač and Šlapetová, 1997
Bohemia, southern part, 1994-96	6		2.9[a]	1.2		Kalač and Šlapetová, 1997
Bohemia, Precambrian shales		7.9±0.3				Řanda and Kučera, 2004
Bohemia, Paleozoic graywache		3.8±0.1				Řanda and Kučera, 2004
Slovenia, Slovenska Bistrica, 1978	1		0.59*	1.1		Byrne *et al.*, 1979
Slovenia, Šalek Valley, 1998-2001	21		4.7			Pokorny *et al.*, 2004
Austria	4	3.2 (1.4-8.1)				Rauter, 1975
Austria, 1975	8	2.8± (1.3-6.7)				Aichberger, 1977
Germany	8	5.7 (1.0-17)				Seeger, 1976
Germany, 1975	26		7.0±0.7	2.2		Seeger, 1976
Switzerland, Vevey, 1972	1	3.2[d]				Stijve and Roschnik, 1974
Switzerland, 1975-82	14	3.5±2.8 (0.41-11)				Quinche, 1983
Yugoslavia, 1972	5	3.7± (3.1-4.8)				Stijve and Cardinale, 1974
Italy, Rome, 1982-83	1	1.3[b]				D'Arrigo *et al.*, 1984
Italy, Reggio Emilia	41	2.7 (1.0-4.3)				Cocchi *et al.*, 2006
Turkey, 1997		0.51±0.20				Sesli and Tuzen, 1999

USA (origin could be international)	6	13±1.9[RSD]				Wuilloud *et al.*, 2004
Contaminateded sites						
Finland, Äestä, chlor-alkali plant, 1-3 km, 1979	3	0.91 (0.23-1.4)				Lodenius and Herranen, 1981
Slovakia, Krompachy, copper smelter, 1990-93		32±19 (max. 63)				Kalač *et al.*, 1996
Slovakia, 1994-96	1		54	2.0		Svoboda *et al.*, 2000
Slovakia, Spiš, Želba Rudñany, 1990-93/97-99	13	30				Zimmermannova *et al.*, 2001
Slovakia, Spiš, Želba Rudñany, 1990-93/97-99	1		19	1.9		Zimmermannova *et al.*, 2001
Slovakia, Spiš, Želba Rudñany, 1997-98	1	55[#]				Svoboda *et al.*, 2000
France, Paris, 1989-90[‡]		34-41				Michelot *et al.*, 1998
Italy, near Siena, cinnabar mining area	1	1.9			2.1[d]	Bargagli and Baldi, 1984
Italy, near Siena, cinnabar mining area	1	0.7			0.5	Bargagli and Baldi, 1984

Notes: No. of samples and no. of specimens (in parenthesis); [+]4.1 μg/g dw in tubes; [#] Summer Bolete *Boletus aestivalis* (*B. recticulatus*); [##]Unpublished data; [a]Without tubes – in tubes was 6.4 μg/g dw; [b]Inorganic 0.79 and organic 0.51 μg/g dw (61 and 39%); [c]CH_3Hg^+ (monomethylmercury) = 0.02 μg/g dw (0.6%); [d]CH_3Hg^+ = 0.04 μg/g dw (0.8%) and BCF_{Hg} = 32; [‡]No information was provided if the region sampled nearby to the city of Paris was contaminated with mercury or not, while concentrations noted are record high; [d]CH_3Hg^+ = 0.26 μg/g dw (13.7%)

A comprehensive dataset on total mercury in King Bolete is in Table 13.2. It seems reasonable to state that the upper limit of mercury for specimens from uncontaminated sites should not exceed 20 μg/g dw in cap or a whole fruit body. In the case of consignment of the mushrooms, pooled sample or a set of the specimens from a particular site the means should not exceed 10 μg/g dw tolerance limit. Dataset of methylmercury in King Bolete is very poor (Table 13.2).

Data on mercury in Bay Bolete are summarized in Table 13.3. Usually total mercury content of caps of Bay Bolete from uncontaminated sites in Poland and elsewhere ranged from 0.11±0.05 to 0.61±0.22 μg/g dw. At two sites were from 0.80±0.50 to 0.81±0.29 μg/g dw, and in one study was 0.073±0.020 μg/g dw. At mercury-contaminated sites, a whole fruit body of Bay Bolete contained this element in concentration around ten-fold greater when compared to caps collected at unpolluted sites (Table 13.3).

Table 13.3. Review of data on concentration, cap to stipe concentration quotient ($Q_{C/S}$) and bioconcentration factor (BCF) values of mercury in Bay Bolete (*Xerocomus badius*) in Poland and other countries (μg/g dw; arithmetic mean, SD, range and median value, respectively)

Country, site and year of collection	n	Fruit body		$Q_{C/S}$	BCF	Reference
		Whole	Cap			
Uncontaminated sites						
Poland, Koszalin; outskrits, 1997-98	15		0.20±0.07 (0.10-0.37)	2.4±0.5	12±11	Falandysz *et al.*, 2004
Poland, Pomorskie Voivodeship, Łubiana, 1994	15		0.22±0.06 (0.10-0.36) 0.21	1.7	13±5	Falandysz *et al.*, 1997
Poland, Wdzydze Lake region, 1996-97	15		0.42±0.45 (0.13-1.9) 0.26	2.5±1.6	11±13	Falandysz *et al.* 2003e
Poland, Wdzydzki Landscape Park, 1995-96	10		0.18±0.05 (0.11-0.28) 0.18	2.7±2.3	28±10	Falandysz *et al.*, 2003b
Poland, Wdzydzki Landscape Park, 1995-96	4		0.12±0.06 (0.11-0.28) 0.10	4.4±2.8	3.9±2.1	Falandysz *et al.*, 2003b
Poland, Zaborski L. Park, 1997-98	10		0.49±0.30 (0.26-1.2) 0.36	1.8±0.6	45±36	Falandysz *et al.*, 2002d
Poland, Trjmiejski L. Park, 1995-96	15		0.81±0.29 (0.47-1.3) 0.72	2.4±1.5	8.0±4.6	Falandysz *et al.*, 2003e
Poland, Mierzeja Wiślana Sand-bar, 1994	15		0.073±0.020 (0.039-0.10) 0.076	1.5	0.44±0.27	Falandysz and Chwir, 1997
Polska, County of Morąg and Łukta, 1997-98	16		0.14±0.08 (0.08-0.31) 0.14	1.9±0.6	7.3±4.8	Falandysz *et al.*, 2003d
Poland, Borecka Forest, 1998	16		0.30±0.07 (0.22-0.44) 0.30	1.3±0.3	13±4	Falandysz *et al.*, 2002c
Poland, Augustowska Forest, 1997-98	16		0.11±0.05 (0.06-0.22) 0.10	1.8±0.4	4.4±2.6	Falandysz *et al.*, 2002a
Poland, Gubin; outskrits 1994	16		0.29±0.16 (0.14-0.76) 0.26	1.8	13±1	Falandysz and Kryszewski, 1996
Poland, Tarnobrzeska Plain, 1995	14		0.80±0.50 (0.28-2.1)0.60	0.84±0.49	180±190	Falandysz *et al.*, 2002
Bohemia, 1986-87	25		0.38±0.28 (max. 1.4)			Kalač *et al.*, 1989 and 1991

Bohemia, 1994-96	14	0.61±0.22 (0.20-1.0)		Kalač and Šlapetova, 1997
Germany	5		0.43	Seeger, 1976
Slovenia, 2001	15		0.24	Pokorny *et al.*, 2001
Switzerland, 73-75			0.44-0.57	Quinche, 1976
Contaminated sites				
Bohemia, lead smelter site, 1987-89	19	0.9±0.4 (max. 2.2)		Kalač *et al.*, 1991
Slovakia, 1990-93/1997-99	20	6.9		Zimmermanova *et al.*, 2001
Slovakia, Krompachy, copper smelter, 1990-93	9	5.2±3.5 (max. 11)		Kalač *et al.*, 1996
Slovakia, 1992-93	4	4.8±3.6		Svoboda *et al.*, 2000
Slovakia, 1997-98	3	3.9±2.4		Svoboda *et al.*, 2000
South Bohemia, Borek, 1997-2000	28	1.3±1.1 (0.1-3.0)		Svoboda *et al.*, 2006

Based on these findings it seems reasonable to conclude, that baseline mercury concentration of a pooled collection of caps of Bay Bolete should not exceed 1.0 μg/g dw, while 2.0 μg/g in a single cap. These values considered could be as threshold value for "uncontaminated" fruit bodies of Bay Bolete, while greater mercury content could imply on mushroom contamination due to elevated Hg content of soil substrate and its pick-up at contaminated areas.

The median values of total mercury content for the fruit bodies of Parasol Mushroom collected recently from 19 sites in Poland ranged from 1.3 to 7.4 in caps and from 0.50 to 4.0 μg/g dw in stipes (Table 13.4) (Falandysz *et al.*, 2007b). The arithmetic means of total mercury in caps from the particular sites were between 1.1±1.0 and 8.4±7.4 μg/g dw (total range from 0.05 to 22 μg/g dw), while in stipes were between 0.53±0.27 and 6.8±7.1 μg/g dw (total range from 0.078 to 20 μg/g dw) (Table 13.4). These mercury concentration data of caps and stipes of Parasol Mushroom differed largely ($0.05 < p < 0.001$; U Mann Whitney test) depending on the site of collection.

In earlier studies in Poland, the means of mercury content for caps of Parasol Mushroom collected at several sites were between 1.1±0.2 and 4.9±3.1 μg/g dw, and maximum concentration noted was up to 13 μg/g dw (Table 13.4). On the European scale but except of the site at the Precambrian shales bedrock in the southern part of Bohemia in Czech Republic, the greatest total mercury concentration of Parasol Mushroom were reported for Bohemia (7.8±0.6 μg/g dw) and Austria (9.0±0.4 μg/g dw) (Table 13.4). For a mentioned the particular site with Precambrian shales bedrock (lack of metallic ores), total mercury content of Parasol Mushroom was 22±1 μg/g dw. Apart from an unpolluted with mercury sites in Europe also mushrooms collected from the polluted areas were examined, and the concentrations reported were up to 200 μg/g dw, what is extremely high (Table 13.4).

Table 13.4. Review of data on concentration, cap to stipe concentration quotient ($Q_{C/S}$) and bioconcentration factor (BCF) values of mercury in Parasol Mushroom (*Macrolepiota procera*) in Poland and elsewhere (μg/g dw; arithmetic mean, SD, range and median value, respectively)

Country, site and year of collection	n	Fruit body		$Q_{C/S}$	BCF	Reference
		Whole	Cap			
Uncontaminated sites						
Finland, Mäntyharju, rural, 1978-79	2	0.96 (0.92-1.0)				Kuusi *et al.*, 1981
Finland, Tikkurila, lead processing area, 1980-81	1	3.6				Liukkonen-Lilja *et al.*, 1983
Poland, Gubin, 1994	16		5.3±0.8 (3.2-6.6)		67±68	Falandysz and Kryszewski, 1996
Poland, Stegna, 1994	15		1.1±0.2 (0.77-1.7)		20±1	Falandysz and Chwir, 1997
Poland, Trjmiejski Landscape Park, 1995	15		4.9±3.1 (1.8-13)		220±120	Falandysz *et al.*, 2003a
Poland, Wieluńska Upland, Pajęczno, 1995	17		4.5±1.7 (2.2-8.2)		170±160	Falandysz *et al.*, 2002b
Poland, Trojmiejski Landscape Park, 1995	8		3.3±1.2 (2.3-6.0)		20±8	Falandysz *et al.*, 2007b
Poland, Nadwarciańska Forest, Jarocin, 1999	12		1.8±0.8 (0.05-2.8)	1.9		Falandysz *et al.*, 2007b
Poland, Iława, 2000	2		2.7 (2.1-3.7)	1.7		Falandysz *et al.*, 2008b
Poland, Stężyca County, Borucino, 2000	4		2.2±0.9 (1.1-3.2) 2.2	4.6±5.1	39±18	Falandysz *et al.*, 2008b
Poland, Kętrzyn, 2000	16		4.3±1.7 (1.1-6.7) 4.6	2.2±0.8		Falandysz *et al.*, 2007b
Poland, Tucholskie Forest, Śliwice, 2000	3		1.1±1.0 (0.05-1.9) 1.3	1.3±0.3	47±46	Falandysz *et al.*, 2008b
Poland, Ełk, 2000	15		1.5±0.3 (1.3-2.6) 1.4	3.2±2.0		Falandysz *et al.*, 2007b
Poland, Bydgoska Forest, 2000	15		1.2±0.7 (0.21-2.6) 1.3	2.4±0.8		Falandysz *et al.*, 2007b
Poland, Lubraniec, 2000	15		2.1±0.6 (1.3-3.5) 2.0	2.2±0.7	16±6	Falandysz *et al.*, 2007b
Mierzeja Wiślana, 2001	10		8.4±7.4 (2.9-22) 5.2	1.4±0.2	19±12	Falandysz *et al.*, 2007b
Poland, Augustowska Forest, 2001	16		4.8±1.1 (3.2-6.8) 4.6	1.8±0.1	190±48	Falandysz *et al.*, 2007b
Poland, County Morąg and Łukta, 2002	9		3.3±0.7 (2.0-4.3) 3.5	1.4±0.3		Falandysz *et al.*, 2007b

Poland, Rafa, 2002	15		3.0±1.1 (1.6-4.8) 2.7	1.3±0.3	180±130	Falandysz *et al.*, 2007b
Poland, Poniatowa, 2002	15		3.1±0.9 (1.8-4.8) 2.9	2.1±0.5	69±23	Falandysz *et al.*, 2007b
Poland, Lidzbark Warmiski, 2003	15		7.4±0.5 (6.3-8.6) 7.4	3.2±1.4	140±14	Falandysz *et al.*, 2007b
Poland, Szczecinek, 2003	15		3.7±0.4 (2.8-4.4) 3.7	1.2±0.2	27±13	Falandysz *et al.*, 2007b
Poland, County Czarne, 2003	16		5.7±1.1 (3.6-8.5) 5.7	1.9±0.1	180±48	Falandysz *et al.*, 2007b
Poland, County Osie, 2003	8		6.0±3.0 (0.56-9.7) 6.0	2.0±1.4		Falandysz *et al.*, 2007b
Poland, Olsztynek, 2003	16		6.5±1.4 (3.8-10) 6.5	1.8±0.1	220±110	Falandysz *et al.*, 2007b
Poland, Szczytno, 2003	16		6.5±2.1 (2.5-9.4) 7.0	1.8±0.4	170±67	Falandysz *et al.*, 2007b
Bohemia (western), Zbiroh, 1996, (unpolluted area)		7.8±0.6				Cibulka *et al.*, 1996
Bohemia (southern; unpolluted area), 1994-96	8	5.0±2.4 (3.0-9.9)				Kalač and Šlapetová, 1997
Czech Republic, České Budějovice, 1986-87	9	5.0±4.4 (1.0-15)				Kalač *et al.*, 1989
Bohemia, Precambrian shales		22±1				Řanda and Kučera, 2004
Germany, southern, 1967-74	5	5.0 (2.1-9.3)				Seeger, 1976
Austria, Dunkelsteiner	4	3.9±0.7				Aichberger, 1977
Austria, Kirchschlag	4	3.1±1.5				Aichberger, 1977
Austria, Ried/Rdmk	3	9.0±0.4				Aichberger, 1977
Austria, Ried/Rdmk	4	5.3±0.4				Aichberger, 1977
Hungary, Las Halim, 1993		1.8±0.1				Vetter and Berta, 1997
Hungary, Bakony, 1993		2.1±0.1				Vetter and Berta, 1997
Hungary, Tanabánya, 1993		2.6±0.0				Vetter and Berta, 1997
Hungary, Tanabánya, 1993		3.5±0.0				Vetter and Berta, 1997
Hungary, Las Kamara, 1994		4.7±0.0				Vetter and Berta, 1997
Hungary, Miskolc, 1994		3.1±0.1				Vetter and Berta, 1997
Hungary, Miskolc, 1994		2.6±0.1				Vetter and Berta, 1997
Hungary, Tanabánya, 1995		2.5±0.1				Vetter and Berta, 1997

Hungary, Las Kamara, 1995		3.0±0.1			Vetter and Berta, 1997
Hungary, Miskolc, 1995		3.2±0.1			Vetter and Berta, 1997
Slovenia, Čemšenik, 1971	1	2.0			Byrne and Ravnik, 1976
Slovenia, Dvór, Dolenjska, rural 1971			6.0[c]		Byrne and Ravnik, 1976
Slovenia, Kurešček, 1971	1	3.5			Byrne and Ravnik, 1976
Slovenia, Šalek Valley, 1998-2001	28	2.3			Byrne and Ravnik, 1976
Italy, Reggio Emilia	15	2.4 (1.5-3.3)[ci]			Cocchi *et al.*, 2006
Spain, Lugo, 1997	6		8.2±11.4 (1.0-29)[h]		Alonso *et al.*, 2000
Spain, Lugo, 1997 (6; rp)	6		1.6±1.4 (0.74-4.3)[rp]		Alonso *et al.*, 2000
Spain, Cordova, 1984	10		1.5±0.7 (0.33-2.7)		Zurera *et al.*, 1986
Contaminated sites					
Slovakia, Krompachy, copper smelter, 1990-93	10	29±25 (max. 94)			Kalač *et al.*, 1996
Slovakia, Rudňany, mercury smelter, 1990-93	3	120±71 (max. 200)			Kalač *et al.*, 1996
Slovakia, Řudňany, mercury smelter, 1990-93	4	31			Svoboda *et al.*, 2000
Bohemia, Choceň, 1996, (slightly contaminated area)		3.3±1.3[w]			Cibulka *et al.*, 1996
Slovakia, Rudňany, mercury smelter, 1997-98	6	41			Svoboda *et al.*, 2000
Slovakia, Rudňany and Krompachy, 1990-93/1997-99	24	44			Zimmermannova *et al.*, 2001
Slovakia, Rudňany and Krompachy, the smelters sites			16[c]		Zimmermannova *et al.*, 2001
Slovakia, Rudňany and Krompachy, the smelters sites			7.9[s]		Zimmermannova *et al.*, 2001
Italy, near Siena, cinnabar mining area, p. 1984	1	4.4[a]		17	Bargagli and Baldi, 1984
Italy, near Siena, cinnabar mining area, p. 1984	1	0.81		6.7	Bargagli and Baldi, 1984

Notes: p. (published); [h](hymefore); [rp](remaining part – without hymefore); [ci]Confidence interval (95%); [a]CH_3Hg^+ = 0.06 μg/g dw (1.4%)

Brown Birch Scaber Stalk from most of the sites surveyed recently in Poland did exhibit total mercury at mean concentrations much smaller, when compared to King Bolete or Parasol Mushroom but similar as noted in Bay Bolete, *i.e.* from 0.38 ±0.23 to 0.64±0.25 μg/g dw (median from 0.36 to 0.65 μg/g dw; Table 13.5). Only for the Gostyńsko-Włocławskie Forest and the County of Starachowice sites the arithmetic means of mercury content for this species were 1.1±0.4 and 1.2±0.4 μg/g dw (media values 1.2 and 1.1 μg/g dw, respectively) (Table 13.5). In stipes of this species, total mercury content was subsequently smaller than in caps. The overall-means of total mercury for 240 caps and stipes of Brown Birch Scaber Salk from the stands in Poland in recent survey were, respectively, 0.63±0.38 (0.072-2.0 μg/g dw) and 0.32±0.20 (0.028-1.2 μg/g dw) (Faladysz and Bielawski, 2007).

Table 13.5. Review of data on concentration, cap to stipe concentration quotient ($Q_{C/S}$) and bioconcentration factor (BCF) values of mercury in Brown Birch Scaber Stalk (*Leccinum scabrum*) in Poland and elsewhere (μg/g dw; arithmetic mean, SD, range and median value, respectively)

Country, site and year of collection	n	Fruit body		$Q_{C/S}$	BCF	Reference
		Whole	Cap			
Uncontaminated sites						
Norway, p. 1978	1		0.19			Allen and Steinnes, 1978
Finland, Mikkeli, 1979	1		0.45			Lodenius et. al., 1981
Sweden, Umeå, 1995	19		0.18±0.13 (0.033-0.47)	1.7±0.8	3.7±4.2	Falandysz *et al.* 2001a
Poland, Gdynia-Orłowo, sea coast site, 1989	4		0.054 (0.023-0.092)			Falandysz *et al.*, 1995
Poland, Gubin, 1994	16		0.29±0.30 (0.12-1.3) 0.20	1.6	9.8±15.3	Falandysz and Kryszewski, 1996
Poland, Kaszuby area, Łubiana, 1994	15		0.37±0.33 (0.093-1.2) 0.27	1.7	3.3±3.2	Falandysz *et al.*, 1997
Mierzeja Wiślana Sand Bar, Stegna, 1994	14		0.29±0.10 (0.14-0.46)	1.6	11±4	Falandysz and Chwir, 1997
Poland, Wieluńska Upland, 1995	15		0.50±0.23 (0.17-0.93) 0.46	1.6±1.8	15±11	Falandysz *et al.*, 2002b
Poland, Tarnobrzeska Plain, 1995	15		0.46±0.32 (0.12-1.4)	2.8±1.2	19±15	Falandysz, 2002
Poland, Trjmiejski Landscape Park, 1996	12		6.7±2.2 (3.2-9.6) 7.3	1.7±1.0	150±58	Falandysz *et al.*, 2003a
Poland, Koszalin, outskrits, 1997-98	15		0.30±0.11 (0.17-0.55) 0.25	1.6±0.5	10±6	Falandysz *et al.*, 2004

Poland, Augusto-wska Forest, 1997-98	16(64)		0.23±0.09 (0.12-0.40) 0.21	2.1±0.6	11±6	Falandysz *et al.* , 2002a
Poland, County of Łukta and Morąg, 1997-98	16(26)		0.70±0.27 (0.14-1.4) 0.46	2.0±0.5	8.7±5.4	Falandysz *et al.*, 2003d
Poland, Zaborski Landscape Park, 1997-98	15	0.33±0.21 (0.13-0.93) 0.24			22±18	Falandysz *et al.*, 2002d
Poland, Borecka Forest area, 1998	16(45)		1.2±0.7 (0.30-2.8)	1.2±0.6	40±16	Falandysz *et al.* , 2002c
Poland, Augustowska Forest, 1998-2001	17		0.65±0.35 (0.12-1.5) 0.65	2.2±0.7	17±6	Falandysz and Bielawski, 2007
Poland, Augustowska Forest, 2000	15		0.48±0.25 (0.19-1.0) 0.44	1.8		Falandysz *et al.*, 2007c
Poland, Wdzydzki Landscape Park, 2000	15		0.46±0.21 (0.13-1.0) 0.41	2.9±1.0	16±10	Falandysz and Bielawski, 2007
Poland, Trjmiejski Landscape Park, 2000	10		0.51±0.19 (0.24-0.77) 0.59	1.7±0.5	15±2	Falandysz and Bielawski, 2007
Poland, Sobieszewska Isl., Gdańsk, 2000	13		0.54±0.26 (0.23-1.1) 0.48	2.4±0.7	19±3	Falandysz and Bielawski, 2007
Poland, County of Kętrzyn, 2000	16		0.43±0.15 (0.26-0.77) 0.39	2.0±0.9	14±6	Falandysz and Bielawski, 2007
Poland, Notecka Forest, 2000	15		0.64±0.25 (0.24-1.1) 0.63	2.8±1.1	18±3	Falandysz and Bielawski, 2007
Poland, Notecka Forest, 2000	15		0.29±0.15 (0.11-0.55) 0.23			Falandysz *et al.*, 2007c
Poland, Sudety Mount., Kłodzka V., 2000	15		0.38±0.23 (0.072-0.76) 0.36	1.8±0.8	14±5	Falandysz and Bielawski, 2007
Poland, Sudety Mount., Kłodzka V., 2000	15		0.25±0.11 (0.07-0.46) 0.26			Falandysz *et al.*, 2007c
Poland, Lubelska Upland, 2000-2001	20		0.58±0.25 (0.29-1.1) 0.53	1.8±0.4	14±5	Falandysz and Bielawski, 2007
Poland, Darżlubska Forest, 2001	15		0.42±0.22 (0.17-0.97) 0.36	1.8±0.6	14±4	Falandysz and Bielawski, 2007
Poland, Tucholskie Forest, Osiek, 2001	15		0.59±0.18 (0.21-1.0) 0.59	1.8±0.6	17±3	Falandysz and Bielawski, 2007
Poland, Gostyńsko-Włocławskie F., 2001	15		1.1±0.4 (0.58-1.7) 1.2	2.3±0.8	20±4	Falandysz and Bielawski, 2007
Poland, County of Starachowice, 2001	14		1.2±0.4 (0.68-2.0) 1.1	1.7±0.4	18±2	Falandysz and Bielawski, 2007
Czech Republic, Bohemia, 1987-89	7	0.7±0.4 (max. 1.4)				Kalač *et al.*, 1991

Czech Republic, Bohemia (south), 1994-96	12	0.28±0.16 (0.13-0.72)	Kalač and Šlapetová, 1997
Contaminated sites			
Bohemia, lead smelter site, 1987-89	20	0.6±0.4 (max. 2.2)	Kalač *et al.*, 1991
Slovakia, Krompachy, copper smelter, 1990-93	6	2.9±3.0 (max. 6.8)	Kalač *et al.*, 1996
Slovakia, Rudňany, mercury smelter, 1990-93	6	15±4.4 (max. 20)	Kalač *et al.*, 1996
South Bohemia, Borek, 1997-2000	10	0.5±0.3 (0.1-1.0)	Svoboda *et al.*, 2006
Germany, former mercury mine area, p. 1995	1	6.2[a]	Fischer *et al.*, 1995

Notes: p. (published); a$CH_3H_g^+$ = 0.08 μg/g dw (1.3%)

In earlier studies of Brown Birch Scaber Stalk in Poland (the fruit bodies harvested in 1989-1998), total mercury content was between 0.054±0.036 and 0.46±0.33 μg/g dw for caps and 0.044±0.011 and 0.35±0.14 μg/g dw for stipes (Falandysz and Kryszewski, 1996; Falandysz and Chwir, 1997; Falandysz, 2002; Falandysz *et al.*, 1995, 1997, 2002a-b, 2003b, and 2004). In two of three other studies, Hg content was somewhat elevated, *i.e.* 0.70±0.27 in caps and 0.35±0.14 μg/g dw in stipes from the Łukta and Morąg County in Poland, while 1.2±0.7 and 1.1±0.4 μg/g dw was for the Borecka Forest region (Falandysz *et al.*, 2002c-d). Evidently highly elevated mercury concentration was found in specimens collected from the Trójmiejski Landscape Park (TLP) in the city of Gdańsk region with a mean value of 6.7±2.3 μg/g dw (median 7.3 and maximum 9.6 μg/g dw) in caps and 4.6±1.7 (median 4.6 and maximum 7.7 μg/g dw) in stipes (Falandysz *et al.*, 2003a). This site in TLP could be contaminated due to nearby embrasure active for several decades of years but also of unknown contamination status because military activities in World War II. No statistically significant relationships occurred between soil pH and organic carbon content and total mercury concentration of caps or stipes of Brown Birch Scaber Stalk (Falandysz and Bielawski, 2007).

In several other studies in Europe total mercury content of Brown Birch Scaber Stalk collected from unpolluted sites ranged from 0.19 to 0.72 μg/g dw (Aichberger, 1977; Allen and Steinnes, 1978; Lodenius *et al.*, 1981; Kojo and Lodenius, 1989;Cibulka *et al.*, 1996; Kala and Lapetova, 1997; Svoboda *et al.*, 2000). Exceptions from this pattern are specimens of Brown Birch Scaber Stalk collected from the mercury polluted regions due to activities of copper and mercury smelting in Slovakia, for which total mercury concentration of the fruit bodies ranged from 2.9±3.0 to 15±4 μg/g dw (years 1990-1993) (Kala *et al.*,

1996). Also highly elevated mercury content showed specimen collected from an old mercury mine site in Germany with 6.2 μg/g dw in cap (Fischer *et al.*, 1995). These examples imply on bioindication ability of Brown Birch Scaber Stalk as regards to soil contamination with mercury, and this feature confirmed is for methylmercury (Fischer *et al.*, 1995).

Fly Agaric is easily recognizable, popular, numerous but poisonous mushroom that in early times in Europe and Euro-Asia found specific use because of its psychedelic properties. The data on total mercury content of Fly Agaric of Poland and elsewhere summarized are in Table 13.6. The ranges of the median values of mercury concentration in caps and stipes of this species, depending on the site in Poland, were from 0.19 to 1.4 and from 0.18 to 0.67 μg/g dw, respectively. The maximum values of mercury concentration in caps and stipes in Poland were 3.3 and 2.3 μg/g dw, respectively. These values are comparable to data noted for Fly Agaric in elsewhere in Europe (Table 13.6). Evidently, at mercury contaminetd sites the fruit bodies of Fly Agaric could accumulate total mercury in great concentration, e.g. 71±9 μg/g dw (Fischer *et al.*, 1995).

Table 13.6. Review of data on concentration, cap to stipe concentration quotient ($Q_{C/S}$) and bioconcentration factor (BCF) values of mercury in Fly Agaric (*Amanita muscaria*) in Poland and elsewhere (μg/g dw; arithmetic mean, SD, range and median value, respectively)

Country, site and year of collection	n	Fruit body		$Q_{C/S}$	BCF	Reference
		Whole	Cap			
Uncontaminated sites						
Norway, p. 1978	1	0.43				Allen and Steinnes, 1978
Norway, mountain region, 1988	1	0.17				Bakken and Olsen, 1990
Sweden , 1995	15		0.39±0.54 (0.0094-1.9)	2.0±1.0	3.8±4.1	Falandysz *et al.*, 2001a
Finland, urban area, 1979	2	0.28 (0.25-0.30)				Kuusi *et al.*, 1981
Finland, 1979	8	0.26±0.04 (0.19-0.32)				Lodenius and Herranen, 1981
Finland, Mikkeli, 1979	5	3.2±4.9 (0.88-12); 0.96*				Lodenius *et al.*, 1981
Finland, Tikkurila, lead smelter area, 1980-81	1	0.50				Liukkonen-Lilja *et al.*, 1983
Finland, Helsinki, 1985-86	1	0.50				Kojo and Lodenius, 1989
Belgium, 1984	1	4.3				Parisis & Heede, 1992
Poland, Mierzeja Wiślana Sand Bar, 1993	15		0.31±0.40 (0.074-0.42)	1.8	35±63	Falandysz and Chwir, 1997

Poland, Wdzydze Landscape Park, 1993	15	0.66±0.18 (0.36-0.95)	1.9	68±37	Falandysz *et al.*, 1997
Poland, Wdzydze L. Park, 1995-96	14	0.096±0.040 (0.057-0.20)	1.6±0.3	32±26	Falandysz *et al.*, 2003b
Poland, Trójmiejski L. Park, 1996/97	15	1.9±1.4 (0.35-5.4)	3.5±1.2	49±46	Falandysz *et al.*, 2003a
Poland, Gubin County, 1994	16	0.64±0.19 (0.22-0.97)	1.6	16±10	Falandysz and Kryszewski, 1996
Poland, Wieluńska Upland, 1995	15	0.55±0.30 (0.15-1.1)	1.7±1.4	21±20	Falandysz *et al.* 2002b
Poland, Borecka Forest, 1998	16 (55)*	0.97±0.28 (0.60-1.6)	1.9±0.7	74±18	Falandysz *et al.*, 2002a
Poland, Łukta County, 1998	16 (48)	0.87±0.22 (0.29-1.0)	2.5±0.7	15±9	Falandysz *et al.*, 2003d
Poland, Zaborski L. Park, 1997-98	17	0.81±0.49 (0.067-1.5)	2.4±1.8	41±27	Falandysz *et al.*, 2001d
Poland, Koszalin, 1997-98	15	0.83±0.29 (0.37-1.4)	2.0±0.5	73±42	Falandysz *et al.*, 2004
Poland, Warmia Land, Morąg, 2000	15	0.39±0.10 (0.19-0.56) 0.35			Falandysz *et al.*, 2007e
Poland, Mazury Land, Giżycko, 2000	15	0.33±0.07 (0.18-0.48) 0.32			Falandysz *et al.*, 2007e
Poland, Bydgoska Forest, 2000	14	0.22±0.20 (0.10-0.90) 0.17			Falandysz *et al.*, 2007e
Poland, Sobieszewska Island, Gdańsk, 2000	8	1.0±1.1 (0.05-3.3) 0.76	1.5±0.8	89±100	Falandysz *et al.*, 2007f
Poland, County of Kętrzyn, 2000	16	0.79±0.33 (0.33-1.4) 0.68	1.9±1.0	38±23	Falandysz *et al.*, 2007f
Poland, Mazury Land, Giżycko, 2000	15	0.76±0.21 (0.63-1.3) 0.68	1.9±0.2	28±14	Falandysz *et al.*, 2007f
Poland, Bydgoska Forest, 2000	15	0.76±0.83 (0.23-3.0) 0.45	1.7±0.5	120±190	Falandysz *et al.*, 2007f
Poland, Lubelska Upland, Poniatowa, 2000	15	0.24±0.13 (0.12-0.48) 0.19	1.2±0.5	8.5±2.1	Falandysz *et al.*, 2007f
Poland, Darżlubska Forest, 2001	15	0.43±0.17 (0.26-0.92) 0.40	1.7±0.8	39±50	Falandysz *et al.*, 2007f
Poland, Wdzydze L. Park, Dziemiany, 2001	15	0.25±0.19 (0.10-0.87) 0.22	1.5±1.5	29±20	Falandysz *et al.*, 2007f

Poland, Tucholskie Forest, Osiek, 2001	15		0.92±0.49 (0.24-2.2) 0.94	3.2±1.5	18±10	Falandysz *et al.*, 2007f
Poland, Tucholskie Forest, Ocypel, 2001	15		1.2±0.3 (0.71-1.8) 1.2	1.8±0.4	46±31	Falandysz *et al.*, 2007f
Poland, Warmia Land, Pasym, 2001	20		0.79±0.28 (0.33-1.3) 0.75	2.4±1.6	36±28	Falandysz *et al.*, 2007f
Poland, Gostyńsko-Włocławskie F., 2001	15		0.63±0.21 (0.31-1.0) 0.58	1.5±0.7	66±85	Falandysz *et al.*, 2007f
Poland, Włocławek and Lubraniec, 2001	15		0.68±0.75 (0.21-2.7) 0.41	1.7±0.4	22±10	Falandysz *et al.*, 2007f
Poland, Nadwarciańska Forest, 2001	15		0.48±0.16 (0.11-0.68) 0.47	2.0±1.4	8.3±4.1	Falandysz *et al.*, 2007f
Poland, Starachowice forests, 2001	15		1.4±0.6 (0.85-2.7) 1.4	2.1±0.6	11±8	Falandysz *et al.*, 2007f
Germany, p. 1976	3	0.73 (0.62-0.80)				Seeger, 1976
Czech Republic, Budziejowice, 1986-87	20	1.6±3.0 (0.17-14)				Kalač *et al.*, 1989 and 1991
Bohemia, northern, Dubi, 1996		0.73±0.33				Cibulka *et al.*, 1996
Bohemia, eastern, Choceň, 1996		1.4±0.1				Cibulka *et al.*, 1996
Bohemia, western, Zbiroh, 1996		11±1				Cibulka *et al.*, 1996
Bohemia, Precam-bian Shales, p. 2004		2.7±0.1[a]				Řanda and Kučera, 2004
Bohemia, uranium/ vanadium ores, p. 2004		4.9±0.2[b]				Řanda and Kučera, 2004
Switzerland, p. 1976	4	0.40 (0.18-0.65)				Quinche, 1976
Switzerland, 1975-1985	17	2.0[#] (0.20-7.3)				Quinche, 1988
Slovenia, Smlednik	1					Byrne *et al.*, 1976
Slovenia, Kuruśćek	1		1.1			Byrne *et al.*, 1976
Slovenia, Dvor, p.1979	1	1.1				Byrne *et al.*, 1979
Croatia, eastern, p.1992	1	0.82				Grgić *et al.*, 1992
Turkey, East Black Sea region, 1997		0.35±0.14				Sesli and Tuzen, 1999
Turkey, East Black Sea region, 2000		0.18±0.04				Demirbaş, 2001

		Contaminated sites		
Finland, Äestä, chlor-alkali plant, 1-8 km, 1979	8	2.4±3.5 (0.39-11)		Lodenius and Herranen, 1981
Germany, Stahlberg, Palatinate	3	71±9 (64-82)[c,d]	0.48-1.0	Fischer *et al.*, 1995

Notes: [#]Median was 1.1 μg/g dm; [*]Number of samples and number of specimens (in parenthese); [a]Precambrian shales; [b]Occurrence of uranium/vanadium ores; [c]Methylmercury 0.63 (0.56-0.71) μg/g dw and [d]young specimen - methylmercury 0.27 μg/g dw, and BCF = BCF 3.0-10; p. (published)

Poison Pax is inedible mushroom. The caps, stipes and the whole fruit bodies of Poison Pax from all sites surveyed in Poland exhibited mercury at relatively small concentration - the median values ranged from 0.01 to 0.10 μg/g dw (caps) and from 0.01 to 0.10 μg/g dw (stipes) (Falandysz and Brzostowski, 2007). The arithmetic means of mercury for most of the sites surveyed ranged from 0.01±0.01 to 0.11±0.06 μg/g dw (caps) and from 0.01±0.01 to 0.11±0.04 μg/g dw (stipes). In this study in Poland Poison Pax from the County of Morąg showed mercury at 0.32±0.25 in caps and 0.09±0.11 μg/g dw in stipes, what is elevated concentration when compared to other sites (Table 13.7). This site is neighbor to the former military testing ground in the County of Łukta. The second site in this study with slightly elevated total mercury content of Poison Pax is localized nearby to an industrialized region of the town of Starachowice with 0.18±0.16 in caps and 0.10±0.03 μg Hg/g dw in stipes (Table 13.7). The median Hg values of mushrooms at the sites showing relatively elevated element concentration due to its asymmetric distribution within the sample sets are usually below the arithmetic means.

Table 13.7. Review of data on concentration, cap to stipe concentration quotient ($Q_{C/S}$) and bioconcentration factor (BCF) values of mercury in Poison Pax (*Paxillus involutus*) in Poland and elsewhere (μg/g dw; arithmetic mean, SD, range and median value, respectively)

Country, site and year of collection	n	Fruit body		$Q_{C/S}$	BCF	Reference
		Whole	Cap			
Uncontaminated sites						
Sweden, Umeå, 1995	13(39)[*]		0.033±0.025 (0.002-0.077)	1.4±1.2	0.16±0.14	Falandysz *et al.* 2001a
Finland, Mikkeli, 1979	2	0.05 (0.030-0.070)				Lodenius *et al.*, 1981
Finland, Helsinki		0.01-0.45				Laaksivorta and Lodenius, 1979
Finland, Helsinki, 1979-84	1	0.03				Kojo and Lodenius, 1989
Poland, Wdzydze Landscape Park, 1994	15		0.024±0.007 (0.013-0.034) 0.024	0.8	2.1±1.0	Falandysz *et al.*, 1997

Poland, Mierzeja Wiślana, Stegna, 1994	15		0.049±0.029 (0.010-0.12) 0.039	1.2	0.91±0.55	Falandysz and Chwir, 1997
Poland, Gubin, 1994	16		0.033±0.10 (0.015-0.059) 0.033	1.1	0.76±0.83	Falandysz and Kryszewski, 1996
Poland, Wieluńska Upland, 1995	15		0.11±0.06 (0.031-0.34) 0.080	1.9±1.1	4.5±4.0	Falandysz *et al.*, 2002b
Poland, Wdzydze Landscape. Park, 1995-96	14		0.029±0.011 (0.005-0.04) 0.030	1.3±1.0	4.5±2.8	Falandysz *et al.*, 2003b
Poland, Borecka Forest region, 1998	16(76)		0.030±0.010 (0.014-0.036) 0.028	1.0±0.3	1.8±0.5	Falandysz *et al.*, 2002c
Poland, Augustowska Forest, 1997-98	16(96)		0.024±0.010 (0.011-0.042) 0.022	1.6±0.4	0.8±0.4	Falandysz *et al.*, 2002a
Poland, Zaborski Landscape Park, 1997-98	15		0.088±0.067 (0.022-0.26) 0.066	1.1±0.5	9.8±12	Falandysz *et al.*, 2002d
Poland, Augustowska Forest, 1999	15		0.03±0.04 (0.02-0.07) 0.03	1.4±0.3	0.75	Falandysz and Brzostowski, 2007
Poland, County of Morąg, 1999	15		0.32±0.25 (0.01-0.71) 0.10	2.5±1.2	6.4	Falandysz and Brzostowski, 2007
Poland, Pomorskie Voivodeship, 1999	15		0.37±0.15 (0.15-0.60)	1.5		Falandysz *et al.*, 2007d
Poland, Sobieszewska Isl., Gdańsk 2000	15		0.01±0.01 (0.01-0.02) 0.01	1.0±0.3	0.2	Falandysz and Brzostowski, 2007
Poland, County of Kętrzyn, 2000	16		0.03±0.03 (0.02-0.03) 0.03	1.7±0.3	1.5	Falandysz and Brzostowski, 2007
Poland, Bydgoska Forest, 2000	15		0.03±0.03 (0.02-0.05) 0.03	1.3±0.3	1.5	Falandysz and Brzostowski, 2007
Poland, Notecka Forest, 2000	15	0.02±0.02 (0.02-0.10) 0.01			0.7	Falandysz and Brzostowski, 2007
Poland, Lubelska Upland, 2000	15		0.04±0.01 (0.04-0.05) 0.04	0.6±1.1	2.0	Falandysz and Brzostowski, 2007
Poland, Sudety Mount., Kłodzka V., 2000	15		0.03±0.01 (0.02-0.05) 0.03	1.2±0.3	0.3	Falandysz and Brzostowski, 2007
Poland, Darżlubska Forest, 2001	15		0.11±0.06 (0.07-0.28) 0.08	1.1±0.4	2.2	Falandysz and Brzostowski, 2007
Poland, Tucholskie Forest, Osiek, 2001	15		0.09±0l.04 (0.05-0.09) 0.08	1.3±0.6	1.3	Falandysz and Brzostowski, 2007

Poland, Tucholskie Forest, Ocypel, 2001	15		0.06±0.01 (0.05-0.09) 0.06	0.6±0.2	1.3	Falandysz and Brzostowski, 2007
Poland, Żuromin, 2001	15		0.04±0.03 (0.02-0.05) 0.04	1.5±0.5	1.0	Falandysz and Brzostowski, 2007
Poland, Włocławek, 2001	15		0.06±0.01 (0.05-0.09) 0.06	1.5±0.4	1.5	Falandysz and Brzostowski, 2007
Poland, Starachowice; outskrits, 2001	15		0.18±0.16 (0.06-0.56) 0.09	1.9±1.5	1.8	Falandysz and Brzostowski, 2007
Contaminated sites						
Finland, Äestä, chlor-alkali plant, 0.1 km	1	4.2				Lodenius and Herranen 1981
Italy, near Siena, cinnabar mining area	1	10			0.13	Bargagli and Baldi, 1984

Notes: *Number of samples and number of specimens (in parenthese)

When compared to the surveys of Poison Pax in Poland similar concentration values of total mercury were noted for specimens collected from the unpolluted sites at outskirts of the city of Umeå in northern part of Sweden. This species there showed mercury at mean concentration of 0.03±0.03 in caps and 0.03±0.02 μg/g dw in stipes (Falandysz *et al.*, 2001a). Also in Finland, total mercury content of Poison Pax reflected baseline concentration of 0.03 and 0.07 μg/g dw at the Mikkeli, while 0.03 μg/g dw and from 0.01 to 0.45 μg/g dw in Helsinki (Laaksovirta and Lodenius, 1979; Lodenius *et al.*, 1981; Kojo and Lodenius, 1989). At the contaminated sites, *i.e.* in proximity to the chlor-alkali plant of Äestä in Finland and at the cinnabar mining site in Italy the whole fruit bodies of Poison Pax contained 4.2 and 10 μg Hg/g dw (Table 13.7).

It is worth noting that one of the problems in mushroom mineral composition study and further data evaluation and comparison are difficulties with sample (carpophore) standardization (size, age and other fungus biotic parameters that could impact chemical elements concentration).

Cap to stipe Hg concentration quotient ($Q_{C/S}$)

The caps of higher fungi usually did contain total mercury at greater concentration than stipes (Tables 13.3-13.7). For King Bolete from thirteen sites in Poland the means of caps to stipes mercury concentration quotient ranged from 1.0±0.7 to 3.2±2.1 (median between 1.1 and 2.7; total range from 0.18 to 9.2) (Table 13.2). At the Olsztyn Lakes site in 6 off 14 King Bolete specimens examined the absolute values of Hg $Q_{C/S}$ varied between 0.18 and 0.50, while median value was 1.4. The Hg $Q_{C/S}$ values for King Bolete in some other studies in Poland were between 1.6±0.4 and 2.5±0.7. In King Bolete collected in other countries, the Hg $Q_{C/S}$ values were from 1.9 to 2.8 (Table 13.2). The tubes are the

morphological part of the King's Bolete cap, which is especially rich in mercury (Byrne *et al.*, 1979; Kalač and Šlapetová 1997). It seems that spores, which are produced and released *via* tubes, could be the richest in mercury part of the mushroom's fruit body. Consequently, a release of spores could result to some degree in depletion of mercury and in time dependent variations of Hg $Q_{C/S}$ values of emerged fruit body.

For Bay Bolete from twelve sites in Poland Hg $Q_{C/S}$ values varied between 1.3 ± 0.3 to 4.4 ± 2.8, and for one site was 0.84 ± 0.49 (Table 13.3). In the case of Parasol Mushroom and depending on the site the median value of Hg $Q_{C/S}$ ranged from 1.2 to 2.7, and for the arithmetic means were from 1.2 ± 0.2 to 4.6 ± 5.1 (total range from 0.18 to 12). In other studies in Poland the means of Hg $Q_{C/S}$ for this species ranged from 1.7 to 2.1 (total range from 0.88 to 2.4) (Falandysz and Kryszewski, 1976; Falandysz and Chwir, 1997; Falandysz *et al.*, 2002b, 2003a). The median value of Hg $Q_{C/S}$ noted for 268 specimens of Parasol Mushroom from all these studies in Poland was 1.8. In a study by Zimmermannová *et al.*, (2001) the value of Hg $Q_{C/S}$ for unspecified number of specimens of Parasol Mushroom collected at contaminated site was 2.0 (Table 13.4). The spores probably are the specific site of elevated mercury accumulation in hymenophore of this mushroom species (Alonso *et al.*, 2000).

In a recent study of Brown Birch Scaber Stalk the Hg $Q_{C/S}$ value for all 240 fruit bodies was 2.1±0.8 (median value 2.0), while range for this parameter between the particular sampling sites was relatively narrow, *i.e.* from 1.7±0.4 to 2.9±1.0 (Table 13.5). In Fly Agaric the mean Hg $Q_{C/S}$ for 204 fruit bodies was 1.9±1.0 (0.16-6.4) (median 1.7), and between the particular sampling sites were from 1.2±0.5 to 3.2±1.5 (the median values from 1.1 to 3.2) (Table 13.6). For 181 fruit bodies of Poison Pax in a recent study the Hg $Q_{C/S}$ value was 1.4±0.5 with range from 0.4 to 5.4, and for the particular sites were from 0.6±0.2 to 2.5±1.2 (Table 13.7).

Hg bioconcentration factor (BCF)

The BCF is as a quotient of total element (or any its species, *e.g.* methylmercury) in cap, stipe or a whole fruit body to soil substratum concentration (Chudzyński and Falandysz, 2007). For caps of King Bolete total mercury BCF values ranged between the sites from 41±6 to 130±39 (total range from 13 to 170), and for the stipes were from 27±18 to 72±22 (total range from 4.0 to 90) (Table 13.2). These values are relatively high but variable depending on the site and confirm high capacity of King Bolete to accumulate mercury.

Parasol Mushroom like King Bolete or some other macromycetes is effective mercury accumulator in the fruit bodies (Falandysz and Chwir, 1997; Falandysz *et al.*, 2002b). The BCF values of total mercury for Parasol Mushroom ranged from 16±6 to 220±110 (total range from 0.52 to 470) for caps and from 7.6±2.6 to 130±96 (total range from 0.52 to 340) for stipes (Table 13.3). An

overall mean mercury BCF value for caps of 184 specimens in recent survey was 110±94 (median 96) and for stipes was 63±68 (median 51) (Falandysz *et al.*, 2007b). These data also confirm that Parasol Mushroom is effective mercury accumulating species.

The BCF values of total mercury for caps of Brown Birch Scaber Stalk collected from many sites in Poland ranged from 14±5 to 20±4 (total mean was 16±5, and median 18), while for stipes were from 6.0±4.0 to 11±1 (total mean was 8.3±3.1, and median 8.1) (Table 13.5). In several previous studies the BCF values of total mercury for caps of Brown Birch Scaber Stalk ranged from 3.7±4.2 to 40±16, and for stipes were from 2.4±2.4 to 40±22 (Falandysz and Kryszewski, 1996; Falandysz and Chwir, 1997; Falandysz, 2002; Falandysz *et al.*, 1995, 1997, 2002b-c, 2003d and 2004). In one study, total mercury BCF vales for caps and stipes of this species collected from the Trójmiejski Landscape Park in Gdańsk were especially high. They reached up to 150±58 and 120±100, on the average, respectively (Falandysz *et al.*, 2003a).

In a recent study of 204 specimens of Fly Agaric the means of BCF value of mercury for caps and stipes were 46±81 (3.0-520) and 33±68 (1.0-380), respectively, and the median values were 20 and 13 (Falandysz *et al.*, 2007f). The median values of Hg BCF for caps of Fly Agaric at the sites with relatively small mercury concentration in soil substrate, *i.e.* where the median values were between 0.010 and 0.034 μg/g dried soil, usually, were between 19 and 51. At more polluted sites with the median concentration values between 0.14 and 0.42 μg/g dried soil, the median values of BCF for caps were between 8.3 and 17 (Table 13.6). A fraction of methylmercury and other mercury compounds in the total mercury content of soils and the fruit bodies of Fly Agarics' from the stands in Poland is unknown, and it complicates judgment on type of relationships between possible mercury species of soil substrate and their bioconcentration potential in fruitbody by this fungus.

Methylmercury when compared to total mercury is much better bioconcentrated by Fly Agaric. A highly elevated total mercury content of humus (62-140 μg Hg/g dw) resulted in great element's concentration of Fly Agaric (64-68 μg Hg/g dw) but low values of BCF (0.48-1.0), while MeHg at 0.05-0.09 μg/g in humus resulted in BCF at 3.0-10 (Fischer *et al.*, 1995).

In the case of Poison Pax a relatively small BCF value of mercury with a median values between 0.2 and 3.3, 0.2 and 2.2 and 0.3 characterized the caps, stipes or whole fruit bodies (Table 13.7). The arithmetic means of Hg BCF for caps and stipes and for whole fruit bodies at some of the sites surveyed were greater, due non-normal distribution of mercury concentration in the sample sets, when compared to the median values. In earlier studies of Poison Pax, noted were some discrepancies in BCF depending on the site. A low (< 1) median BCF value for caps and stipes of specimens collected from the Vistula River sand-bar, the Augustowska Forest, and the town of Gubin outskirts, also did indicate on exclusion but not bioconcentration (BCF > 1) of mercury by this species. But at

some other stands for this species (the Wieluńska Upland, Wdzydze and Zaborski Landscape Parks, Borecka Forest, County of Łukta and Morąg sites) a relatively greater median BCF values, which varied between 1.6 and 6.0 for caps and between 1.7 and 5.5 for stipes, did indicate a somehow on its bioconcentration potential (Table 13.7) (Falandysz *et al.*, 2002a).

In a recent survey, very low BCFs of mercury were found for Poison Pax specimens collected from the stands poor in organic carbon. At the Sobieszewska Island on the south coast of the Gulf of Gdańsk (0.6-0.7% C in soil) and of the Kłodzka Valley in the Sudety Mountains (1.3-3.5% C in soil) the median BCF values for this species varied between 0.2 and 0.3 for caps and between 0.2 and 0.2 for stipes (Table 13.7). Nevertheless, also low BCF value of 0.3 characterized the fruit bodies collected from the stand rich in humus (6.7% C in soil) in Notecka Forest site (Table 13.7). In these studies (Falandysz and Brzostowski, 2007), poor in humus soils could contain less methylmercury when compared to soils richer in organic carbon. This feature could have an impact on total mercury BCF values noted.

The bioconcentration of mercury by some mycorrhizal fungi was dependent on the content of sulfhydryl, disulfide and methionine containing proteins in the fruit bodies (Kojo and Lodenius, 1989). Aichberger and Horak (1975) reported that BCF values of mercury in Champignon Mushroom (*Agaricus bisporus*) were independent of the substrate element concentration. In a study by Bressa *et al.* (1988) when $HgNO_3$ was added to the straw compost containing corn combs, sawdust and $CaCO_3$ at concentrations between 0.05 and 0.2 μg Hg/g dw, and subsequently inoculated with the mycelia of Common Oyster Mushroom (*Pleurotus ostreatus*), BCF value of mercury in the fruit bodies was between 65 and 140. Inorganic mercury (^{203}Hg) added to substrate is bound mainly by high-molecular-weight protein fractions in the fruit bodies of Champignon Mushroom and Common Oyster Mushroom (Lasota *et al.*, 1990; Lasota and Florczak, 1991).

The value of BCF of mercury, any other chemical element, or its chemical form accumulated in the fruit bodies could characterize accumulation potential of the species. The BCF value of chemical element for given fungus that is relatively high, constant and independent of its soil content, at least for a given range of concentrations, certainly would imply on perfect bioindication potential of the species. Nevertheless, there is consensus that higher fungi are not suitable as bioindicators of soil contamination with metallic elements or metalloids.

As observed for experimental or real nature condition, small mercury concentration in substrate implies on higher BCF value and *vice versa*. For example, the rate of mercury uptake by lignocellulytic *Agrocybe aegerita* was high even when the concentration in the substrate was small (Brunnert and Zadrazil, 1983). This phenomenon is found usually also among wild grown macromycetes (Falandysz *et. al.*, 2007a-d) (Tables 13.2-13.7).

So far, only in two studies positive relationships between mercury in fruit bodies of wild grown fungi and soil were noted. In one report methylmercury

(MeHg) accumulated at 0.08-0.24 μg/g dw in three specimens of Bay Bolete and specimen of Brown Birch Scaber Stalk correlated with MeHg content of humus (0.01-0.03 μg/g dw) but no such relationship occurred for total Hg (Fischer *et al.*, 1995). Methylmercury was effectively bioconcentrated by these two fungi (BCF, 9.6-12), while total mercury not (BCF, 0.17-1.7). In another study positive correlation was observed between total mercury in caps and stipes (0.77-1.7 and 0.41-0.83 μg/g dw) of Parasol Mushroom and humus (0.037-0.093 μg/g dw) underneath to the fruit bodies-element speciation was not determined (Falandysz and Chwir, 1997).

Hg intake with mushrooms and possible Hg tolerance limits for mushrooms

Wild grown edible mushrooms are popular food items traditionally used in kitchen of many nations worldwide. It is hard to assess amount of wild grown mushrooms eaten *per capita* daily, weekly or on a longer term by general population, mushroom fanciers or people with easy access (villagers; forest workers) to this kind of food. No mention about availability of consumption rates exclusively for any specific mushroom species consumed. Based on the human feeding realities wild grown mushrooms on the average are only small portion of daily diet on a longer term. Certainly, among mushroom (several species) dish fanciers in rural and nearby to forest settlements in northern part of Poland consumption rate of mushrooms (calculated on fresh weight) sometimes could be up to 10 kg *per capita* annually for some individuals, depending on family gourmet tradition, seasonal abundance of mushrooms but also income. Kala and Svoboda (2000) informed that in Czech Republic 72% of the families' pick-up wild grown mushrooms and the average yield is 7 kg *per familia* annually, while an individual consumption rate of these mushrooms could reach 10 kg *per capita*.

Mercury and methylmercury accumulated to great concentration in edible mushroom could create hygienic problem. To assess possible risk due to intake of mercury accumulated in fruit bodies a reference dose (RfD; 0.3 μg Hg/kg body weight daily) and value of Provisionally Tolerable Weekly Intake (PTWI; 5 μg Hg/kg bw) (JECFA, 1978; US EPA, 1987) could be applied. PTWI for methylmercury is 1.6 μg/kg bw (JECFA, 2007).

As noted, mercury content of King Bolete varies depending on the sampling sites' geographical location (Table 13.2). King Bolete surveyed recently in Poland, when grown up at the background areas contained mercury in caps at mean concentration up to 7.6±3.1 μg Hg/g dw, and in an individual fruit body were up to 14 μg Hg/g dw (Falandysz *et al.*, 2007a, 2008). These values are largely similar to the maximum mercury concentrations found earlier in King Bolete at the background areas in Poland or elsewhere in Europe. Based on dataset from Table 13.2, it seems reasonable to state that the upper mercury limit for the King's Bolete specimens from uncontaminated sites should not exceed 20 μg/g dw in a cap or a whole fruit body, while for consignment of mushrooms, pooled sample or a given site mean concentration should not exceed 10 μg/g dw.

The specimens of King Bolete from the majority of the sites surveyed exhibited mercury at concentration around five-fold below 20 or 10 μg/g dw, respectively. Methylmercury fraction of the total mercury content of a few specimens of King Bolete examined was very small, i.e. 0.6 and 0.8% in two studies, and in another study was 39% (Table 13.2).

In addition, King Bolete is very rich in selenium (Falandysz, 2006, 2008). Selenium as selenocysteine is essential for selenocysteine-containing enzymes including selenoprotein P and glutathione peroxidases in mammals. These selenoproteins and glutathione function as antioxidant defense including also protection against absorbed mercury. Hence, it seems that from the point of view of food toxicologist or nutritionist mercury content of King Bolete could be consider at least depending also on content and interactions between methylmercury, inorganic mercury and selenium.

Using criteria similar as in the case of King Bolete, for Bay Bolete (Table 13.3), the tolerance limit of mercury should not exceed 5 μg/g dw in cap or a whole fruit body, while for consignment of mushrooms, pooled sample or a given site mean concentration should not exceed 1 μg/g dw.

The cap of Parasol Mushroom is edible and stipe is inedible. Based on existing database (Tables 13.3) it seems reasonable to state, that upper food hygienic limit (tolerance) for mercury content of Parasol Mushroom at the uncontaminated sites should not exceed 25 μg/g dw in a single cap, while on the average, for pooled sample or consignment, should not exceed 10 μg/g dw per site. An exception can be the background areas with specific type of the parent bedrock geochemistry (e.g. Precambrian shales bedrock). For such sites, total mercury content of pooled sample of caps of Parasol Mushroom on the average could slightly exceed limit of 10 μg/g dw. A record high mean 120±71 μg/g dw value of total mercury accumulated in the fruit bodies of Parasol Mushroom is for the specimens collected from the mercury smelter area, and up to 200 μg/g dw was found in a single specimen (Kalac *et al.*, 1996). Nevertheless, knowledge on highly toxic methylmercury content and its fraction in total mercury content of the caps of this species is lacking.

Total mercury data of caps of Brown Birch Scaber Stalk in recent survey of many sites in Poland, when converted to fresh weight (assuming 90% water content), were from 0.038 to 0.12 μg/g wet weight on the average (total range from 0.0072 to 0.20 μg/g ww) (Falandysz and Bielawski, 2007; Falandysz *et al.*, 2007c). A cap of this species is usually the appreciated part of fruit body, and contains total mercury at greater concentration when compared to stipe. Methylmercury could form only 1.3% fraction of total mercury in Brown Birch Scaber Stalk's fruit body, as reported by Fischer *et al.* (1995). Based on a "worst case" scenario (total mercury content 2.0 μg/g dw) weekly consumption of up to 1.5 kg fresh caps of Brown Birch Scaber Stalk originating from the unpolluted sites in Poland will not result in exceeding of the limit for maximum weekly total mercury intake.

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CHAPTER - 14

ALCOHOL OXIDASE ENZYME OF METHYLOTROPHIC YEASTS AS A BASIS OF BIOSENSORS FOR DETECTION OF LOWER ALIPHATIC ALCOHOLS

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Introduction

Quick and accurate detection of alcohol content is relevant for many fields of human activity. This type of analysis is necessary for the chemical and enzymatic alcohol production, product composition control in food industry, development of alcohol-based pharmaceutical preparations, clinical and toxicological studies, biodiesel and bioethanol production, and environmental monitoring (Mao *et al.*, 2008). A variety of methods and strategies had been reported for determination of the content of lower aliphatic alcohols (C_1 – C_6) including: gas chromatography, liquid chromatography, refractometry, spectrophotometry based on NADH detection (Azevedo *et al.*, 2005). Although some of these methods are precise and reliable, they are complex and time consuming, require previous separation processes (distillation, evaporation), expensive instrumentation, and trained operators. Such disadvantages can be overcome by using enzymatic (including biosensor) methods. The previous works presented thorough analysis of application of two enzymes, alcohol dehydrogenase and alcohol oxidase (AO) for the analysis of aliphatic alcohols (Glazkov *et al.*, 1997;

Mizgunova *et al.*, 1998). Actually, the methods described there are a basis of many currently existing biosensor approaches.

The methods of precise and quick analysis of alcohol content are an up-to-date problem. Due to some features of biosensor analysis (simplicity, reliability, specificity and sensitivity), the development and application of biosensors for the analysis of alcohol-containing samples is thought to be promising. Enzyme sensors for ethanol or methanol detection may be based on alcohol oxidase, NAD+ -dependent alcohol dehydrogenase, or pyrroloquinoline quinone (PQQ)-containing alcohol dehydrogenase immobilized on the corresponding transducer.

This chapter puts an emphasis on consideration of the analytical parameters and peculiarities of biosensor detection of ethanol and methanol of practical relevance. The application of alcohol oxidase from yeast cells for this purpose is considered in preference. The data on alcohol dehydrogenase are given only in the case when they extend the notions of biosensor application of enzymes for alcohol detection. Since biosensor and biofuel cells in essence are the types of instruments with the common principle of measurement, the data on alcohol application in biofuel cells are presented. Practical application of alcohol oxidase based biosensors is considered.

Alcohol oxidase properties used for alcohol detection

Alcohol oxidase (AO, Alcohol oxidoreductase, EC 1.1.3.13) is the key enzyme for methanol metabolism in methylotrophic yeast species, including *Pichia pastoris*, *Hansenula polymorpha* and *Candida boidinii*. As a rule, commercial AO is obtained from the same cultures. In yeast cells, the enzyme catalyses the first step of methanol catabolism, namely its oxidation to formaldehyde, with concomitant production of hydrogen peroxide according to the reaction:

$$CH_3OH + O_2 \xrightarrow{AO} HCHO + H_2O_2$$

Other lower alcohols including ethanol are oxidized by the same scheme. In vivo, the protein is compartmentalized into special cell organelles termed peroxisomes. Some fungal cultures are known to be sources of aryl-alcohol oxidases (Varela *et al.*, 2001; D'Annibale *et al.*, 2005). Aryl-alcohol oxidase is an extracellular flavoenzyme involved in lignin biodegradation by some white-rot fungi. The enzyme catalyzes the extracellular oxidation of aromatic alcohols to the corresponding aldehydes. Some fungi also contain AO that catalyzes oxidation of primary alcohols (Alvarado-Caudillo *et al.*, 2002), but they are not used for alcohol detection.

Native, enzymatically active AO is a homo-octameric flavoenzyme of approximately 600 kDa, which consists of eight identical subunits, each containing one non-covalently bound flavin adenine dinucleotide as a cofactor (Vonck and van Bruggen, 1990). In AO, during the catalytic reaction the cofactor is reduced to the hydrogenated form ($FADH_2$) and then reoxidized to the native

form by molecular oxygen with the formation of hydrogen peroxide. In consideration of the above, alcohol oxidase-based biosensors have an advantage when compared with those using alcohol dehydrogenase as a biorecognition element, because the latter needs the cofactor to be added to the sample or immobilized on the sensor surface.

Irrespective of the source, native alcohol oxidase has the maximal activity at methanol oxidation, though the Michaelis-Menten constants and, accordingly, the activity at oxidation of different alcohols significantly depend on the source of the enzyme. Some of the biochemical data on AO isolated from *Hansenula polymorpha* (taken as an example) are as follows: Michaelis-Menten constants for different substrates are (in mM) 33.3 for allyl alcohol, 26.6 for benzyl alcohol, 6.6 to 16.2 for ethanol, 2.6 to 10.5 for formaldehyde, 0.4 to 2.15 for methanol, 166 for n-butanol, and 66 for n-propanol. Inhibitors of activity AO are 1,4-butynediol, 4-hydroxy-2-butynal, cyclopropanol, cyclopropanone, formaldehyde, H_2O_2, hydroxylamine, KBr, KCN, methanol, NaN_3, propargyl alcohol, propynal, and urea. The pH range of observed activity is 5.5 to 8.5. AO is stable in solution for at least 6 months at 4°C (http://www.brenda.uni-koeln.de/).

AO is used for the qualitative or quantitative determination of ethanol or methanol as a component of test kits, for biosensor development, for alcohol removal, aldehyde or hydrogen peroxide production, and oxygen scavenging. AO is also known to be used for detection of formaldehyde according to the reaction (Dmytruk *et al.*, 2007):

$$HCHO + O_2 + H_2O \xrightarrow{AO} HCOOH + H_2O_2$$

Since the AO enzyme converts all primary alcohols and formaldehyde, it exhibits the lack of selectivity to ethanol. However, this should not be a problem at application of such a biosensor for the analysis of ethanol in natural samples such as wine, since ethanol is present in much higher concentrations.

The biosensor principle of alcohol measurement consists in direct registration of the level of oxygen or hydrogen peroxide in the region of immobilized protein or registration of the current induced by the charge transfer by artificial electron acceptors.

Immobilization

In the course of biosensor development, an important step is the stage of fixation of enzyme molecules, because the sensitivity and reproducibility of detection of low-molecular compounds depend a lot on the method of immobilization. Among other conducting polymers, polypyrrole (Ppy) was found to possess attractive applications as a fundamental building material. Polypyrrole is often used in the design of various analytical tools. For the first time, the self-encapsulation of redox enzyme, glucose oxidase (GOX), into polypyrrole matrix was shown in the works (Ramanavicius *et al.*, 2005; Ramanavicius *et al.*, 2006b).

Pyrrole polymerization was initiated by the catalytic effect of hydrogen peroxide released at glucose oxidation. The presence of entrapped glucose oxidase within polypyrrole was determined by basic application of polypyrrole-coated glucose oxidase nanoparticles (GOX/Ppy) in electrochemical biosensor design. More than tenfold increase of Michaelis-Menten constant K_m was determined for polypyrrole-coated GOX as compared with the native GOX. The authors believe that the considered process of self-encapsulation will occur in other oxidase-based systems as well. It should be noted that we have not yet encountered any works on alcohol oxidase immobilization by the above mechanism. The work indicates that other oxidases are predicted for various bioanalytical purposes and other biocatalytic applications (e.g., with AO-based systems). This work was continued in the study of (Ramanaviciene *et al.*, 2006). The latter was devoted to the methods of formation of conducting polypyrrole nanoparticles formed at using GOX as an initiator of polymerization. Polymerization was performed in a solution consisting of GOX, glucose, and pyrrole. Ppy formation was conditioned by the release of hydrogen peroxide at glucose oxidation. The formation of polypyrrole was monitored in two ways: by a spectrophotometer and by an atomic force microscope. The authors have shown that the shape of clusters and other Ppy characteristics may change depending on the type of substrate. It is suggested that this method of Ppy formation may be useful for creation of bioanalytical structures. The generalizing data on immobilization of biologically active molecules within Ppy during electrochemical deposition can be found in the review (Ramanavicius *et al.*, 2006c). Such unique properties of this polymer as prevention of some undesirable electrochemical interactions and facilitation of electron transfer from some redox enzymes are discussed.

The method of electrochemical immobilization includes electrosynthesis of a film from the monomers and the enzyme on the electrode surface (Badea *et al.*, 2003). The method has a single stage and takes little time. The enzyme is fixed in the polymer film by means of binding reagents, e.g., glutaraldehyde. Non-conducting electropolymerized films are a selective barrier, which does not let through electroactive interfering components basing on the charge of size of molecules. Immobilizaiton in electropolymerized films has been used for development of biosensors on the basis of glucose oxidase, lactate oxidase, oxidase of L-amino acids, and alcohol oxidase. Glucose oxidase immobilized in the polymer layer in the course of polymer formation in the presence of glutaraldehyde on the surface of platinum electrode was stable for 60 days. AO immobilized in the same way made it possible to detect ethanol in the linear range of 0.25 - 5 mM; the effect of ascorbic acid was 1.85%.

The immobilization of *Hansenula polymorpha* alcohol oxidase onto controlled-pore glass beads has been considered in the works (Azevedo *et al.*, 2004a; Azevedo *et al.*, 2004b). Mini packed-bed AO bioreactors were used to monitor ethanol concentration. The horseradish peroxidase and its reducing substrates were used to eliminate the inactivating effect of hydrogen peroxide on the enzyme. This strategy led to high operational stabilities (more than 10 h with

no loss in conversion degree) and was successfully applied in a flow injection analysis system for ethanol analysis. The effect of various polymers on AO stability has been studied. Polyethylene glycols decrease the stability. With dextrans, stability depends more on the charge of the polymer than on its molecular weight. Polyethylenimine also protects AO against thermal inactivation.

The thermal stability of AO from different sources was examined (Lopez-Gallego *et al.*, 2007). Enzymes from *Candida boidinii*, *Hansenula* sp. and *Pichia pastoris* were immobilized through covalent and ionic adsorption techniques. The enzyme from *Hansenula* sp. was the most stable of the three. The stability of the enzymes was strongly dependent on the enzyme concentration, suggesting that the first inactivation cause could be subunits dissociation. The enzymes covalently immobilized in glyoxyl agarose had the highest stability. Dextran aldehyde was used to stabilize the quaternary structure of these enzymes. The use of dextran aldehyde increased stability of the enzymes but reduced their activity. The other immobilization technique for both enzymes was the ionic adsorption on agarose coated with polyethylenimine. The ionic adsorption of the enzymes in polyethylenimine agarose made it possible to stabilize the quaternary structure of the enzymes. At the same time, the activity recovered was about 50% for both enzymes under study, while the activity recovered of the enzymes immobilized in glyoxyl agarose followed by the treatment with dextran aldehyde was slightly more than 10%.

The new method of covalent immobilization of AO on magnetic beads of 50-75 μm and 75-150 μm in diameter is developed. Magnetic beads are prepared in the presence of glycidyl metacrylate and methyl metacrylate through polymerization of the suspension in the presence of a cross-linker, ethylene dimethyl metacerylate. The enzyme activity on microspheres of lesser diameter was 4.8-fold higher as compared with the larger particles. Operational stability of immobilized AO was 30 measurements per day. Storage stability was examined for 60 days. In the first 30 days, the activity decreased insignificantly; on day 60, the activity decreased by 80% (Kiralp *et al.*, 2008).

The new type of conducting polymer has been proposed in the work (Yildiz and Toppare, 2006). Immobilization of tyrosinase and alcohol oxidase is achieved in the copolymer of pyrrole with vinyl alcohol with thiophene side groups. The kinetic parameters (maximum reaction rates (V_{max}) and Michaelis–Menten constants (K_m)) are obtained. V_{max} and K_m are found as 2.75 μmol/(min electrode) and 18 mM for copolymer of pyrrole with vinyl alcohol and thiophene/alcohol oxidase electrode. The decrease of AO activity at examination of operational stability was 30%; the drop of activity of immobilized tyrosinase was 15%. The loss of activity at the storage of immobilized AO was 80% during 30 days; the decrease of stability at the storage of immobilized tyrosinase was 40%. The operational and storage stabilities show that, although they have limited storage stabilities, the enzyme electrodes are useful with respect to operational stabilities.

The disadvantage of AO-based biosensors with hydrogen peroxide detection is rather high working potential, +600 mV, in relation to the reference electrode. In the work (Smutok *et al.*, 2006), a biosensor has been developed on the basis of AO and horseradish peroxidase immobilized trough electrodeposition paints. The Os-complex provided low working potential of 50 mV. The biosensor was formed using platinized graphite electrodes. The linear range of ethanol and methanol detection was 0-2 mM and 0-1 mM, respectively. Operational stability of the biosensor was 1000 measurements at a drop of response value by 50%; storage stability was 16 days at a drop of the current value by 50% on day 14 of storage. The concentrations of ethanol in the 1000 times diluted wine samples of Cabernet-Sauvignon, Chardonnay, and Kheres-strong were obtained. The measured concentrations were compared with the values obtained from the same samples using gas chromatography and enzymatic test kit "Alcotest" as reference methods. The values obtained by the developed bi-enzyme biosensors exhibited good correlations with the standard methods.

Amperometric biosensor on the basis of AO from *Hansenula polymorpha C-105* immobilized by the method of electrochemical polymerization has been developed (Shkotova *et al.*, 2005). Commercial platinum screen-print electrodes were used as transducers. The basis of polymer matrix was commercially available resin Resydrol AY 498 w/35WA purchased at Slutia GmbH, Austria. Biosensors were characterized by good reproducibility and operational stability with the lower detection limit 8×10^{-5} M. Ethanol detection in wine samples and in the must was performed for 4 samples of wines obtained at the Institute of Grapes and Wine "Magarach" and in 29 samples of wine materials from different fermentation stages. A good correlation (r = 0.995) of the results of ethanol detection in wine and during fermentation by the amperometric biosensor and by standard methods (densitometry and enzyme kit "Alcotest") was obtained.

Let us consider a few examples of using NAD^+ -dependent alcohol dehydrogenase and PQQ-containing alcohol dehydrogenase immobilized on the corresponding transducer. The interest in alcohol dehydrogenase (ADH) instead of AO in alcohol sensor fabrication is due to the fact that oxygen is not involved in the reaction. The reaction of ethanol oxidation in this case proceeds by the following scheme:

$$CH_3CH_2OH + NAD^+ \xleftrightarrow{ADH} CH_3CHO + NADH + H^+$$

It is interesting that the authors have used a rarely occurring method of enzyme immobilization in carbon paste. Poly(o-phenylenediamine) was electropolymerized on the surface of an alcohol dehydrogenase-nicotinamide adenine dinucleotide-modified carbon paste electrode to yield an electrochemical biosensor for ethanol. The amperometric response to ethanol was obtained by means of the electrocatalytic oxidation of the enzymatically produced NADH, at an applied potential close to 0 V (Ag/AgCl). The linear range of ethanol detection was $3 \times 10^{-8} - 3 \times 10^{-6}$. Response time was 20 s. The following trend in sensitivity was observed: ethanol > l-propanol > amylalcohol = l-butanol > 2-

propanol > 2-butanol > ethylenglycol > methanol > glycerol. The biosensor was used for ethanol detection in alcoholic beverages. The diluted cider, table wine and whisky samples yield well-defined amperometric responses similar to those of ethanol standards. All samples were compared to the enzymatic-spectrophotometric method and the distillation method. The correlations with the spectrophotometric and distillation methods were excellent. The biosensor assay requires only sample dilution and takes a few minutes as opposed to the conventional procedure taking much more time (Alvarez-Crespo *et al.*, 1997).

The amperometric biosensor for ethanol detection has been constructed using the modern method of immobilization in nanobiocomposite film (Lee and Tsai, 2008). The nanobiocomposite film based on multi-walled carbon nanotubes, a chitosan binder, and alcohol dehydrogenase (EC 1.1.1.1, 316 U mg^{-1}, from *Saccharomyces cerevisiae*) was immobilized on a glassy carbon electrode. The system operated under the potential of +0.7 V versus Ag/AgCl. NAD^+ was added to phosphate buffer solution for ethanol detection. The optimized biosensor shows a sensitivity of 0.1646 A M^{-1} cm^{-2} and an apparent Michaelis–Menten constant of 0.38 mM. The authors have shown the biosensor to have an extremely high sensitivity (detection limit of 0.52 μM) and an equally short lifetime: the initial level of the signal decreased by 15% in 30 min of sensor operation.

Biosensors and their practical application

Application of alcohol oxidase

Enzyme biosensors based on oxygen level registration; optical biosensors; conductometric biosensor; measurement of real samples

Oxygen registration

AO-based biosensors recording the level of oxygen in a bioreceptor commonly have the electrochemical principle of detection. Clark-type electrodes (Kitova *et al.*, 2004; Azevedo *et al.*, 2005; Wen *et al.*, 2007) and three-electrode schemes with an open working electrode are widely used for this purpose. The work of the authors (Wu *et al.*, 2007a) is interesting due to using the three-electrode scheme for registration and nanomaterials for immobilization. Carbon nanomaterials possess good electrical conductivity, unique structural and catalytic properties, high loading of biocatalysts, good mechanical and chemical stability, and excellent adsorption and penetrability; thus, they can be used for biosensor manufacturing. A biocomposite film based on thionine-functionalized carbon nanofiber and alcohol oxidase was formed on the electrode surface. Based on the excellent catalytic activity of the biocomposite film toward reduction of dissolved oxygen, a sensitive ethanol biosensor was produced. Electrochemical measurements were performed with a conventional three-electrode system

comprising platinum wire as auxiliary electrode, saturated calomel electrode as reference electrode, and modified glassy carbon electrode as working electrode. The applied potential was –0.4 V. The biosensor detected ethanol in the linear range of 2 to 252 μM. The coefficient of variation was 5.0-5.9%. The study of the biosensor stability showed that the signal value decreased during 4 weeks by 4 % only.

Iron-porphyrin has excellent electrocatalytic properties toward the detection of many biochemical analytes. A highly sensitive biosensor has been developed on the basis of biofunctional hybrid nanocomposite of carbon nanofiber with water-soluble iron(III) mesotetrakis(*N*-methylpyridinum-4-yl) porphyrin. The nanocomposite combined the good conductivity of carbon nanofiber and the excellent catalytic activity of both carbon nanofiber and iron-porphyrin complex toward the reduction of dissolved oxygen. The glassy carbon electrode was coated in series with the nanocomposite and then AO. Electrochemical measurements were performed using the three-electrode system at the potential of the working electrode relative to the reference electrode of 200 mV. The linear range of ethanol detection was 2-112 μM of ethanol. The sensor response value on day 30 after the manufacture was 91% of the initial value (Wu *et al.*, 2008).

Inclusion of enzymes into biocompatible biomaterials is an effective method of improving the activity and stability of immobilized enzymes. Wen *et al.* (2007) showed that eggshell membrane increased the lifetime of the enzyme. The eggshell membrane contains mainly protein fibers: a natural biopolymer, which is biocompatible with the enzymes and has a high permeability towards enzyme substrates. In the biosensor for ethanol detection, AO was immobilized on the eggshell membrane using chitosan. The Clark-type oxygen electrode was used as a transducer. The linear range of ethanol detection was 60 μM – 0.6 mM; the lower detection limit was 30 μM; response time was 1 min. The optimal enzyme and chitosan concentrations were 1 mg and 0.3% (mass/volume), respectively. During 3 months of storage, the activity decreased by 13%. The biosensor was tested on beer, liquor, and strong beverage samples. The results of biosensor analysis had a high correlation with the method of gas chromatography.

Optical biosensors

Entrapment of the enzyme within hydrophilic polymer is a common technique for construction of a biphase biosensor operating in organic solvents. The drawback of the method is retardation of biocatalytic reactions because of the low efficiency of substrate and product transportation through the hydrogel network. For overcoming this disadvantage, in one of the previous works Wu and Choi have proposed a new method of enzyme immobilization based on mixing the hydrogel structure with entrapped enzyme and other components with small hydrophobic particles. An optical alcohol biosensor consisting of alcohol oxidase and horseradish peroxidase coimmobilized in a spongiform hydrogel matrix in conjunction with an optical oxygen transducer has been successfully fabricated.

This method has a number of advantages. First, the hydrogel membrane becomes thinner and increases the interfacial contact area. Second, immobilization matrix including hydrophilic and hydrophobic structures is highly permeable for hydrophilic and hydrophobic organic solvents. Third, the diffusional path through the thin membrane is shortened (Wu and Choi, 2004). This approach is further developed in the work of Wu *et al.* (2007). The optical principle of oxygen detection has been used. The biosensor for alcohol detection in the organic phase was based on AO, horseradish peroxidase, and an oxygen transducer. The enzymes were jointly immobilized in a spongiform hydrogel matrix based on hydroxethyl carboxymethyl cellulose, an adduct of 3-methoxy-4-ethoxy benzaldehyde, 4-tert-butylpyridinium acetohydrazone, silica gel particles, and octadecylsilica particles in conjunction with an optical oxygen transducer. The optical sensing is based on oxygen quenching on the fluorescence intensity of tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) didodecyl sulphate adsorbed on silica gel particles. The biosensor detected methanol in the range of 2.3 – 90 mM in *n*-hexane. Response time was 4.5 and 7.5 min for 60 and 10 mM, respectively. Operational stability of the biosensor was more than 45 measurements; lifetime was over two weeks. The high accuracy of biosensor assay of methanol in motor gasoline samples containing methanol up to 20% of the volume was shown (Wu *et al.*, 2007b).

Conductometric biosensor

It should be noted that the conductometric principle of measurement is used rather rarely at biosensor development. The cause of this fact is not fully clear. Probably, these biosensors are low-selective. Their main advantage of the approach is that almost all enzymatic reactions involve either consumption or production of charged species and hence lead to a global change in the ionic composition of the tested sample and, consequently, electric conductivity. This example presents the parameters of a conductometric biosensor based on AO for detection of formaldehyde. Construction of the biosensor is very simple. The transducer was represented by interdigitated thin-film planar electrodes and immobilized AO from *Hansenula polymorpha*. The biosensor reached steady-state response in 1 min. The range of formaldehyde detection was 0.05 to 500 mM. The biosensor developed was not completely specific and selective. It was also sensitive to primary alcohols and some other substrates. The disadvantage of the biosensor was higher response to the formaldehyde/methanol mixture as compared with the response to formaldehyde. Operational stability was no less than 20 h; relative standard deviation appeared to be about 3%; storage stability was more than 1 month (Dzyadevych *et al.*, 2001).

Measurement of real samples

The question important for the practice, which is associated with biosensor construction, is the search of the methods of directed modification of catalytic properties of the enzymes, in particular, due to the change of Michaelis-Menten

constant K_m. Taking into account that the ethanol concentration in wine is far outside the working range of the sensor (around 12% (v/v), representing approximately 2.06 M ethanol), a 2000- to 400,000-fold sample dilution is necessary prior to the analysis to adjust sample concentration to the linear range of the biosensor. Thus, a considerable error may result from this dilution step, which can be circumvented by a calibration procedure recording the response obtained from the same sample solution after variation of the dilution ratio (Niculescu *et al.*, 2002). In this connection, it is preferable to take measures for decreasing biosensor sensitivity. The problem is of both practical and fundamental significance. Its analysis for oxidases generating hydrogen peroxide has been performed in the work (Ramanavicius *et al.*, 2008b). The study was carried out with glucose oxidase selected as a model object. The enzyme was immobilized on a carbon rod electrode by cross-linking with glutaraldehyde. The effect of abrupt increase of the K_m value (from 5.8 to 250 mM) was caused by subsequent formation of a conducting layer of polypyrrole entrapping GOX. An increase in the upper detection limits was estimated by calculation of K_m. A significant increase in the long-term stability of the electrode modified by GOX and Ppy was detected as compared with that of unmodified one. Further application of this approach for other oxidases is predicted for biosensors that require the extension of detection limits. As regards the measurement of alcohol content in drinks, including strong beverage, it may be supposed that this approach probably gives an opportunity to perform measurements without dilution.

Three mediators (4-ferrocenylphenol (FP), 2-ferrocenyl-4-nitrophenol (FNP), and N-(4-hydroxybenzylidene)-4-ferrocenylaniline (HBFA)) based on ferrocene were studied for the possibility of being combined with coenzyme PQQ of glucose and alcohol dehydrogenases on the carbon electrode surface. A screen-printed carbon electrode (SPE) suitable for ADH and glucose dehydrogenase immobilization served as a transducer. The biosensors were used for ethanol and glucose measurements in beverages. There was a good correspondence ($r = 0.978$ for glucose and $r = 0.920$ for ethanol) between the data obtained by means of biosensors, on the one hand, and by the refractometric or hydrometric methods, on the other hand. The operational stability of biosensors is determined by inactivation of immobilized enzymes rather than by leakage of a mediator from an electrode (Razumiene *et al.*, 2003).

Lapa *et al.* (2003) developed an on-line sequential injection analysis system using amperometric detection for the simultaneous monitoring of glucose and ethanol in fermentation media. The method is based on glucose and ethanol oxidation catalyzed by the corresponding oxidases: glucose is oxidized to gluconic acid and hydrogen peroxide in the presence of glucose oxidase and ethanol is oxidized to acetaldehyde and hydrogen peroxide in the presence of alcohol oxidase. The hydrogen peroxide (H_2O_2) produced was monitored amperometrically at a constant potential. GOX and AO were immobilized on controlled-pore glass using glutaraldehyde for immobilization and connected to

the measurement system in parallel as two independent electrodes. Measurements were performed at a potential of +600 mV using the three-electrode system. The linear range for ethanol was within 0.15 to 30 mg dm^{-3}. The system was applied for the monitoring of glucose and ethanol in beer fermentation. The maximum relative deviation between the results obtained by the developed system and the conventional method was below 4.3% for glucose and 4.4% for ethanol (Lapa *et al.*, 2003).

The quantitative estimation of ethanol and acetaldehyde in exhaled air must be performed to determine the state of alcoholic intoxication. In this respect, great attention has been paid to the development of semiconductor gas sensors. However, the selectivity of semiconductor sensors yields to the selectivity of enzyme-based biosensors. Mitsubayashi *et al.* (2003, 2005, 2008) developed two types of bioelectronic gas sensors (bio-sniffers) including AO and ADH for detection of ethanol and acetaldehyde in exhaled air, respectively. The sniffer devices for gaseous ethanol (alcohol oxidase, amperometric and optic analysis) and acetaldehyde (amperometric analysis of enzymatic reactions) were constructed through immobilization of the enzymes on electrodes covered with filter paper and a hydrophilic membrane, respectively. The detection range was 1- 500 ppm for ethanol vapor and 0.11-10 ppm for acetaldehyde at amperometric detection (Mitsubayashi *et al.*, 2003; Mitsubayashi *et al.*, 2005; Mitsubayashi *et al.*, 2008).

A new biosensor system based on carboxyl esterase and alcohol oxidase has been developed. The biosensor was intended for aspartame detection in soft drinks and commercial sweetener tablets. The sensor was a bienzyme system composed of carboxyl esterase and alcohol oxidase from *Candida boidinii*, immobilized in gelatin membrane, subsequently combined with the dissolved oxygen electrode. Aspartame is first cleaved by esterase to L-Asp-L-Phe and methanol. Methanol is then oxidized by alcohol oxidase. The linear range of aspartame concentrations was 5.0×10^{-8} and 4.0×10^{-7} M. Assay time was 10 min (Odaci *et al.*, 2004).

The methodology of methanol detection in biodiesel based on the flow analysis and membrane-based extraction has been proposed (Araujo *et al.*, 2008). A hydrophilic membrane was used for liquid–liquid extraction in the system with an organic sample fed to the donor side of the membrane and the methanol transfer to an aqueous acceptor buffer solution. The quantification of methanol was then performed in aqueous solution with the joint use of immobilized AO, soluble peroxidase, and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid). Biodiesel samples from different sources were then directly analyzed without any sample pre-treatment. Statistical evaluation showed a good compliance, for a 95% confidence level, between the results obtained with the flow system and those furnished by the gas chromatography reference method. The proposed methodology turns out to be more environmentally friendly and cost-effective than the reference method.

Biosensors based on hydrogen peroxide registration

The principle of biosensor detection of alcohols using AO can also be based on registration of hydrogen peroxide formed in the reaction of ethanol oxidation by alcohol oxidase. In its turn, hydrogen peroxide is most often registered by electrochemical or spectrophotometrical methods. The advantage of the electrochemical method of detection is the absence of dependence of the results on the oxygen content in a sample. The concentration of hydrogen peroxide is linearly connected with the analyte concentration. A disadvantage is the sensitivity to a number of electroactive components oxidized in this reaction.

Alcohol oxidase, glucose oxidase, lactate oxidase, L-aminoacid oxidase, glycerol-3-phosphate oxidase, lysine oxidase, and choline oxidase were immobilized on Prussian blue layer supported on 1,2-diaminobenzene nanotubules onto Pt electrodes (Curulli *et al.*, 2004). The conductive polymer nanotubules and the Pt electrodes were a basis for supporting highly dispersed Prussian blue, which acts as an active component for H_2O_2 detection. The glucose biosensors assembled with nanostructured poly(1,2-diaminobenzene) showed the detection limit of 5×10^{-5} M, the wide linearity range of $5 \times 10^{-5} - 5 \times 10^{-3}$ M, high selectivity, and stability for 3 months at 4°C and at least 4 weeks at room temperature. Similar analytical parameters and stability were also studied for ethanol, L-(+)-lactic acid, L-leucine, glycerol-3-phosphate, lysine, and choline biosensors.

Recently, SPE have been widely used as transducers of biosensors. Their advantage is the low cost and possibility of bulk production of biosensors. The work (Boujtita *et al.*, 2000) describes SPE electrodes based on the paste containing 5% cobalt phthalocyanine and coated with a semipermeable membrane. For ethanol analysis, the carbon electrode modified with cobalt phthalocyanine was further modified by drop-coating with 5 μl of AO solution (1 U/μl) and left to dry for 4 h. The adsorbed enzyme film was covered with a membrane (nitrocellulose acetate or polycarbonate membrane) attached by super-glue. Ethanol detection was based on the measurement of a signal produced by hydrogen peroxide, a product of the enzymatic reaction. The linear range of ethanol detection at the potential of +400 mV relative to the silver chloride electrode was 0-1.5 mM. Chronoamperometry was used to measure the ethanol concentration in beer following a simple dilution step. The determination was performed ten times with a fresh biosensor for each measurement; the mean concentration was 4.98% and standard deviation was 7.63%. The manufacturer's specification is 5% for this particular product, which indicates that the biosensor gives reliable results.

The novel original method of selective detection of ozone has been presented, which is performed using the AO-based biosensor (Stergiou Dimitrios *et al.*, 2006). The principle of this method consists in the quick selective sorption of ozone by eugenol (4-allyl-2-methoxyphenol) folowed by detection of the formed formaldehyde. Ozone adds rapidly to the double bond of the allyl group

of eugenol, which has been immobilized onto a hydrophobic C-18 reactor and the so produced formaldehyde is collected into the working buffer solution and pumped to the detector. The enzyme was applied onto a nylon membrane, dried, and placed onto the electrode between the internal acetate-cellulose and outer polycarbonate membranes. The formed hydrogen peroxide was registered at a potential of +0.65 V relative to the reference electrode. The linear range of ozone detection was 3-200 μg/ml; the lower detection limit at Signal/Noise = 3 was 1.1 μg/ml. The biosensor allowed more than 100 measurements without the loss of activity; storage stability was over 2 months.

Mediator biosensors

The biosensors of mediator type contain an artificial electron acceptor to avoid the effect of electrochemically active admixtures and oxygen on the response. The chemistry of the reactions in them is as follows: the enzyme enters into the redox reaction with the substrate and then is oxidized by the mediator; the mediator, in its turn, is oxidized on the electrode.

Composite graphite-Teflon electrodes based on AO (EC 1.1.3.13, from Pichia Pastoris, activity 1430 U ml^{-1}), horseradish peroxidase, and ferrocene mediator have been developed for the monitoring of alcohol content in alcoholic beverages (de Prada *et al.*, 2003). The bienzyme electrodes are constructed by simple physical inclusion of the enzymes and the mediator in the bulk of graphite-70% Teflon rigid cylindrical pellets. The working potential of the electrodes was zero Volts. The storage stability of the enzymes without the loss of activity was 3 months. The linear ranges of methanol and ethanol detection were 1×10^{-5} - 4×10^{-4} M and 1×10^{-4} - 4×10^{-3} M, respectively. The biosensor was employed to determine the ethanol content in local beer (4.8 vol. % ethanol), alcohol-free beer, local red (12%) and white (12%) wine, and in two different commercial liquors. Only dilution in the phosphate buffer background solution was needed as sample treatment in all cases, in order to set the analyte concentration in a sample within the linear range of the calibration plot. The possibility of using the bienzyme composite electrode in HPLC was demonstrated by performing the separation of methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol. The authors have also applied the HPLC method, using the bienzyme electrode as amperometric detection system, for determination of methanol in the wine samples, where it may be present in a small amount as a product of pectines degradation, and in the liquor samples, where it may be present even at a concentration of 3.5 g l^{-1}.

The self-assembly technique has been very widely used to construct the ordered molecular layers possessing various functions. An amperometric biosensor based on immobilized AO (isolated from *Hansenula* sp.), horseradish peroxidase, and electron transport mediator has been developed using the method of self-assembly. The biosensor was designed for methanol detection in plant extracts. The self-assembled mixed monolayer of 4,4'-dithiodibutyric acid covalently attached two enzymes and 11-ferrocenyl-1-undecanethiol as an

electron mediator on an gold electrode. Methanol was detected in 0.1 M sodium phosphate buffer, pH 6. The linear range of methanol detection was 10 – 150 μM for the three-electrode measurement scheme at a potential of 200 mV and 0-500 μM for mini-sized biosensor allowing the measurement in a 96-well assay plate system. The authors have successfully quantified methanol in low-purity tobacco plant (*Nicotiana tabacum*) extracts by the biosensor showing sensitivity comparable to that of gas chromatography/mass spectrometry. Response time was 2 min. AO had a high sensitivity to methanol; the response to ethanol was 8.9%; an insignificant sensitivity to 1-propanol was observed (Hasunuma *et al.*, 2004).

It is well known that AO is incompatible with the most of free-diffusing mediators, which makes it extremely difficult to design electron-transfer pathways between the redox active center of the enzyme and the electrode at low potentials. Barsan and Brett (2008) have shown for the first time that poly (neutral red) can mediate the enzymatic reaction catalyzed by AO. Poly (neutral red) was electropolymerizsed on carbon film electrodes. AO from *Hansenula polymorpha* was immobilized by cross-linking with glutaraldehyde in the presence of bovine serum albumin. Measurements were performed at a potential of – 0.3 V versus saturated calomel electrode. The lower detection limit was 30 μM of ethanol. The linear detection range was 0 – 0.7 mM; relative standard deviation was no more than 8.6%. After three weeks of operation, biosensor responses were 58% of the initial level of the signal. The electrodes were stored in the buffer solution at 4°C. Sensitivity decreased by 12% in 6 weeks of storage. The high level of selectivity towards common interferences provides the possibility of using this device in the clinical, environmental and food control. The performance of the biosensor for practical applications in the analysis of natural samples was demonstrated by performing determination of the alcohol content in red and white wines, estimated in terms of ethanol concentration. The samples required only a simple dilution step. The biosensor accuracy was assessed by comparison with the results given by the wine producers (Barsan and Brett, 2008).

It is known that the available biosensors reach saturation at very low concentrations alcohols, which results in the necessity of sample dilution. The work (Dmytruk *et al.*, 2007) describes alcohol detection on the basis of mutant AO forms from the strains of *Hansenula polymorpha* resistant to allyl alcohol in methanol medium. Their application extends the linear detection range. The bioselective layer of the sensor included two parts: the inner, with horseradish peroxidase electrochemically precipitated in the presence of carboxylate-containing polymer modified with an osmium-pyridyl complex (*Os-Ap59*), and the outer, with AO from mutant strain CA2 immobilized via cathodic precipitation in the presence of an amino-containing polymer. The Os-containing polymer served as a redox mediator in the electrochemical reaction. Platinized graphite rods were used as working electrodes, which were sealed in a glass tube using epoxy thus forming disk electrodes. The enzymes had lower affinity to

substrates at maintenance of the maximal reaction rate. The values K_m for the AO of the wild type and two mutant strains were 0.62, 2.48, and 1.1 mM, respectively. The amperometric biosensor based on the mutant AO form had an extended linear range of detection towards ethanol (0 – 4 mM) and formaldehyde (0 – 1 mM) as compared with native AO.

Quinohemoprotein alcohol dehydrogenase from *Gluconobacter sp.* was used to create a biosensor for ethanol detection. The biosensor is constructed by cross-linking a quinoprotein alcohol dehydrogenase to an Os-complex-modified poly(vinylimidazole) redox polymer using poly(ethylene glycol) diglycidyl ether. Graphite rods were used as basis electrodes. Working potential was +300mV vs. Ag/AgCl. The optimized sensor showed a sensitivity of $0.336\pm0.025 AM^{-1}$ cm^{-2} for ethanol and a detection limit (calculated as three times the signal-to-noise ratio) of 1 μM. The optimal biosensor configuration was applied for analysis of the ethanol content in wine. The obtained value was 12.2 vol.%, being in good agreement with the concentration declared by the producer (12 vol.%) (Niculescu *et al.*, 2002).

The essence of studies Tkac *et al.* (2007) is creation of nanocomposite electrodes, i.e. electrodes based on biomolecules and nanoparticles/nanotubes for recognizing targeted molecules in wine. Enzyme-based devices have an Achilles heel – low storage stability at ambient temperature, which requires chilled shipment and storage. The reason for choosing a nanocomposite for preparing electrodes is driven by an objective to produce extremely stable and selective bioelectrodes. The stability of enzymes inside the matrix is enormous (e.g., months at ambient temperature). The idea of connecting a traditional winemaking technology with modern nanotechnology is attractive: combining the art of winemaking with the power of nanotechnology (Tkac *et al.*, 2007).

Application of microbial cells with alcohol-oxidizing activity

Biosensors for alcohol detection are developed with the employment, along with the enzymes, of the cells of microorganisms which have a number of advantages: (a) high stability, as they are the whole where all metabolic systems are in "protected", naturally optimized state; (b) inexpensive as compared with enzymes and immunoglobulins and do not need labor-consuming purification processes; and (c) are capable of multistage transformations without introduction of additional exogenous factors. The amperometric biosensor based on the cells of *Candida tropicalis* obtained in logarithmic phase and containing AO is presented in the work (Akyilmaz and Dinckaya, 2005). Ethanol determination is based on the assay of the differences in the respiration activity of the cells in the absence and presence of ethanol. The cells were immobilized in gelatin with addition of glutaraldehyde (0.1%). In the optimization studies of the microbial biosensor, the most suitable microorganism amounts were found to be 4.42 $mg.cm^{-2}$. The response of the microbial sensor linearly depended on ethanol concentration in the range of 0.5 – 7.5 mM; response time was 2 min. The following substrate specificity of the microbial biosensor was obtained and

expressed in the percentage of responses to ethanol: ethanol, 100; methanol, 59; propanol, 21; butanol, 21; *iso*-propanol, 0; etylenglycol, 0; dietylenglycol, 0 (for substrate concentration 2.5 mM).

The development of analytical instruments based on homogenized tissue using plant tissue materials is a relatively new development in the biosensor technology. In this study (Akyilmaz and Dinckaya, 2000), a homogenized mushroom (*Agaricus bisporus*) tissue based electrode with the alcohol oxidase activity was developed. Enzyme was immobilized with gelatin and cross-linking agent glutaraldehyde on dissolved oxygen probe for detection of ethyl alcohol. The electrode response depends linearly on ethyl alcohol concentration between 0.2 and 20 mM. It has been established that the optimized variant provides for the measurement in phosphate buffer, pH 7.5, 50 mM, at 35°C. By using the electrode prepared, it was possible to make 60 measurements in a 10-h period, and response time of the electrode was 2 min for each measurement.

Bacteria of the genus *Gluconobacter* contain PQQ-dependent dehydrogenases, including PQQ-containing alcohol dehydrogenase. PQQ-dependent dehydrogenases are characterized by the broad spectrum of oxidized substrates; hence, both the enzymes and the bacteria are a prospective basis for biosensor development. The work (Tkac *et al.*, 2002; Tkac *et al.*, 2003) presents a microbial biosensor based on *Gluconobacter oxydans* cells for ethanol detection, obtained through surface modification of ferricyanide (ferricyanide mediated) glass-carbon electrode. Selectivity of the *Gluconobacter oxydans* based biosensor to ethanol was significantly improved by using a cellulose-acetate membrane that has a limited permeability for glucose. The cellulose-acetate membrane increased the ratio of "ethanol/glucose" sensitivity 58.2 times and "ethanol/glycerol" sensitivity 7.5 times as compared with the dialysis membrane. The lower limit of ethanol detection was 0.85 μM (S/N=3). The selectivity of the biosensor toward alcohols was better compared to previously published enzyme biosensors based on alcohol oxidase or alcohol dehydrogenases. The biosensor was used for the on-line monitoring of ethanol in the course of periodic fermentation of glucose by immobilized cells of *Saccharomyces cerevisiae* at the initial glucose concentration of 200 g/l.

Biofuel cells

Production of electric energy from inexpensive stuff is one of the important trends of modern power engineering. These types of stuff include nondrinkable ethyl/bioethyl alcohol produced in considerable amounts. For its application, biological fuel cells based on alcohol oxidase, NAD^+-dependent alcohol dehydrogenase or PQQ-containing alcohol dehydrogenase, and microbial cells are manufactured. In biological fuel cells, the reactions on one or both electrodes are performed biocatalytically at a moderate temperature and pressure. An essential aspect is development of maximally simple and effective constructions. So, the work (Ramanaviciusa *et al.*, 2005) considers a biofuel cell based on the enzymes that perform a direct exchange of electrons with the electrodes. The

biological material for the anode was the quino-hemoprotein-alcohol dehydrogenase (QH-ADH) from the bacterium *Gluconobacter* sp. 33; the cathode contained co-immobilized glucose oxidase from *Aspergilus niger* and microperoxidase 8 from the horse heart (MP-8). Two enzymes, GOX and MP-8, were acting in a consecutive mode. Hydrogen peroxide oxidized by MP-8 was accepting electrons directly from the carbon electrode. The maximal open circuit potential 270 mV was reached in the presence of a concentration over 2 mM for both substrates (ethanol and glucose). One more original solution has been proposed in the work (Ramanavicius *et al.*, 2008a). This work considers the biofuel cell construction with ethanol as a fuel for both the anode and the cathode. Electron transport mediators are not used. The anode is represented by immobilized QH-ADH, while the cathode consists of co-immobilized alcohol oxidase and microperoxidase MP-8. Two enzymes, AO and MP-8, acted in the consecutive mode. The ability of QH-ADH to transfer electrons directly towards the carbon-based electrode and the ability of MP-8 to accept electrons directly from the same type of electrodes was exploited in this biofuel cell design. Direct electron transfer to/from the enzymes was a basis for generating an electric potential between the anode and cathode. Application of immobilized enzymes and the same type of fuel for both electrodes makes it possible to avoid the compartmentation of enzymatic biofuel cell. The maximal open circuit potential of the biofuel cell was 240 mV.

Table 14.1. Analytical parameters for the considered alcohol oxidase-based biosensors

Principle of detection, transducer	Method of AO immobilization	Lower limit and range of detection	Operational/ storage stability	Application	Reference
Detection of H_2O_2 at +400 mV. Screen-print electrodes	AO was applied to a carbon electrode modified by cobalt phthalocyanine and coated with a semipermeable membrane	0-1.5 mM of ethanol	Disposable electrodes	Ethanol content in beer	Boujtita *et al.*, 2000
Conductometric enzyme electrode based on interdigitated thin-film planar electrodes and immobilized AO	Membrane was formed by cross-linking of AO with bovine serum albumin in saturated glutaraldehyde vapour. DEAE-dextrane was added	0.05-500 mM of ethanol	20 h (a drift rate of about 2.5% h^{-1}); 1 month	Detection of formaldehyde	Dzyadevych *et al.*, 2001
Platinum electrode; detection of H_2O_2 at +650 mV versus Ag/AgCl	Immobilization in electropolymerized films	0.25 - 5 mM of ethanol	---	---	Badea *et al.*, 2003
Detection of H_2O_2 at +600 mV, three-electrode system	AO was immobilized on controlled-pore glass using glutaraldehyde	0.15 - 30 $mg \times dm^{-3}$ of ethanol	---	Ethanol monitoring at beer fermentation	Lapa *et al.*, 2003

Optical oxygen transducer. Detection in organic phase	Immobilization in hydrogel matrix	0.08 – 90 mM of methanol in *n*-hexane	60 measurements (80%); 2 weeks (55%)	Commercial mixture of methanol-gasoline	Wu and Choi, 2004
Dissolved oxygen electrode	AO immobilized in gelatin membrane	5.0×10^{-8} and 4.0×10^{-7} M	---	Detection of aspartame	Odaci *et al.*, 2004
Platinum screen-printed electrodes	Immobilization by the method of electrochemical polymerization	8×10^{-5} M of ethanol	---	Wine samples, process of wine fermentation	Shkotova *et al.*, 2005
Platinum electrode; dete-ction of hydrogen peroxide at +650 mV	AO was applied to a nylon membrane and placed on the electrode between the inner acetate-cellulose and outer polycarbonate membranes.	1.1 µg/ml; 3-200 µg/ml of ozone	100 measurements (100%); 2 months (100%)	Ozone monitoring	Stergiou *et al.*, 2006
Platinized graphite electrodes. Detection of hydrogen peroxide at -50 mV	AO and horseradish peroxidase were immobilized by electrodeposition paints using Os-complex.	0-2 mM of ethanol	1000 measurements (50%); 14 days (50%)	Wine samples	Smutok *et al.*, 2006
Three-electrode system; reduction of O_2 at – 0.4 V	AO was immobilized on carbon nanofiber modified by thionine	2-252 µM of ethanol	4 weeks (96%)	---	Wu *et al.*, 2007a
Oxygen electrode	AO was immobilized on egg-shell membrane using chitosan	30 µM; 60 µM – 0.6 mM of ethanol	36 measurements (100%); 3 months (86.6%)	Wine samples	Wen *et al.*, 2007
– 0.3 V relative to saturated calomel electrode. Poly (neutral red) was used as a mediator	AO was immobilized by cross-linking with glutaraldehyde in the presence of bovine serum albumin	30 µM; 0 – 0.7 mM of ethanol	3 weeks (56.7%); 6 weeks (88%)	Wine samples	Barsan, Brett *et al.*, 2008
Fiber optic oxygen sensor	An optical bio-sniffer. Immobilization of AO onto a tip of a fiber optic oxygen sensor; an oxygen sensitive ruthenium organic complex	0.71 - 51.49 ppm of ethanol vapor	---	Monitoring of the gaseous ethanol	Mitsubayashi *et al.*, 2003
Bioelectronic gas sensors (bio-sniffer). Carbon and Ag/AgCl electr-odes (AO); Pt electrodes (ADH).	Immobilization of AO and ADH on electrodes. AO or ADH was mixed with photo-crosslinkable poly (vinyl alcohol) having stilbazolium groups	1 – 500 ppm of ethanol; 0.11–10 ppm of acetaldehyde	---	Estimation of ethanol and acetaldehyde in exhaled air	Mitsubayashi *et al.*, 2005

Pt electrodes, +700 mV vs. Pt	Formaldehyde dehydrogenase was immobilized onto the sensitive area of the Pt-electrode membrane. FALDH was mixed with photocrosslinkable poly (vinyl alcohol) having stilbazolium groups.	40 - 3000 ppb	---	Formaldehyde detection in the gas phase. Environmental analysis.	Mitsubayashi *et al.*, 2008
Modified glassy carbon electrode; working potential 200 mV versus Ag/AgCl	The electrode was coated with a nanocomposite of carbon nanofiber with water-soluble iron(III) mesotetrakis (*N*-methylpyridinum-4-yl) porphyrin and with AO	2 – 112 mM of ethanol	30 days (91%)	Estimation of ethanol in beer	Wu *et al.*, 2008

Biosensor development is directly connected with the construction of biofuel elements. A potential-generating biosensor is actually a model of a biofuel cell. The work (Ramanavicius *et al.*, 2006a) has examined a new construction of potentiometric biosensor. The biomaterial was the enzyme QH-ADH from *Gluconobacter* sp. 33. The enzyme immobilized on the carbon rod electrode by glutaraldehyde generated electrochemical potential at oxidation of ethyl alcohol based on the ability to separate charges and transfer electrons via established intrinsic electron transfer chain towards carbon surface. The potential value was in the range of 115 – 190 mV. The potentiometric signal was more stable than the amperometric signal based on the same QH-ADH-modified carbon electrode. The ability to directly generate electric potential opens up new possibilities for the application of QH-ADH and other direct electron transfer exhibiting enzymes in the design of new potentiometric sensors, biofuel cells, and self-powering bioelectronic devices.

The total analytical parameters for the considered alcohol oxidase-based biosensors is presented in Table 14.1.

Conclusion

The chapter considers the data on application of AO for development of biosensors for the detection of lower aliphatic alcohols. The most part of AO-based ethanol biosensors belongs to the first generation of biosensors, i.e., they rely on the detection of O_2 or H_2O_2. Certain progress has been made in the course of intensive search of mediators, but their number is still insignificant. Further advances can be expected from a third generation-type of biosensor, i.e. the one that features direct electronic communication between an enzyme and an electrode. Probably, the hope is coupled here with application of nanomaterials. Determination of the tertiary and quaternary structure of AO would also allow the identification of the more labile regions of the protein, which could then be the focus of a rational stabilization strategy in order to increase the enzyme stability and the half-life of AO-based biosensors. At the same time, it should be

noted that the modern level of practical AO application is rather high and substantially simplifies the solution of many important problems of alcohol detection.

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CHAPTER - 15

CHITIN METABOLISM IN FUNGI

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Introduction

The fungal kingdom is very diverse, with species growing as unicellular yeasts to branching hyphae. Developing an outer protective layer, namely the cell wall, is critical for growth and survival of the fungal cell in the diverse environments where fungi live. The shape and integrity of the fungus is dependent upon the mechanical strength of the cell wall, which performs a wide range of essential roles during the interaction of the fungus with its environment. The wall composition frequently varies markedly between different species of fungi (Adams, 2004). The fungal cell wall is a complex structure composed of chitin, glucans and other polymers, and there is evidence of extensive cross-linking between these components. The complexity of the cell wall means that its biogenesis demands a significant number of cellular activities that have to act in concert with the essential functions controlling cell growth and morphogenesis, since the wall determines cell shape. Furthermore, the wall is a highly dynamic structure subject to constant change, for example, during cell expansion and division in yeasts, and during spore germination, hyphal branching and septum formation in filamentous fungi. Cell wall polymer branching, cross-linking, and the maintenance of wall plasticity during morphogenesis, may depend upon the activities of a range of hydrolytic enzymes found intimately associated with the fungal cell wall (Dahiya, 2007).

Chitin is one of the main components of fungal cell wall. The chitin of fungi posses principally the same structure as the chitin occurring in other organisms. However, a major difference results from the fact that fungal chitin is associated with other polysaccharides, which do not occur in the exoskeleton of arthropods. Furthermore, occurrence of chitosan is apparently restricted to fungi (Peter, 2004). The molecular mass of chitin in fungi is not known. However, it is

estimated that bakers'yeast synthesizes rather uniform chains containing 120-170 GlcNAc monomer units.

Chitin Structure

Chitin is an unbranched polymer of N-Acetyl-D-glucosamine. It may be regarded as a derivative of cellulose, in which the hydroxyl groups of the second carbon of each glucose unit have been replaced with acetamido (-NH(C=O) CH_3) groups (Fig. 15.1). Chitin polymers tend to form microfibrils of 3 nm in diameter that are stabilized by hydrogen bonds formed between the amine and carbonyl groups.

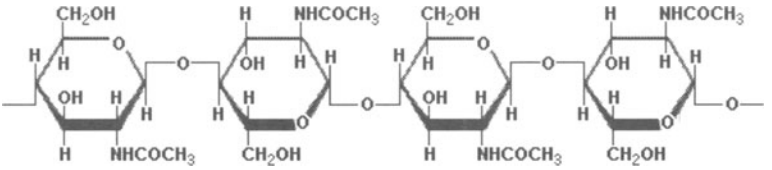

Fig. 15.1 Structure of chitin.

X-ray diffraction analysis suggested that chitin is a polymorphic substance that occurs in three different crystalline forms, termed α-, ß- and γ-chitin. They mainly differ in the degree of hydration, in the size of the unit cell and in the number of chitin chains per unit cell (Kramer and Koga, 1986). In the α-form (Fig. 15.2), all chains exhibit an anti-parallel orientation; in the ß-form (Fig. 15.3) the chains are arranged in a parallel manner; in the γ-form sets of two parallel strands alternate with single anti-parallel strands. Among these α-chitin is the most abundant form of chitin.

Chitin synthesis and its regulation

Chitin synthesis is a process maintained across the fungal kingdom. Chitin synthesis is based on the regulation of distinct chitin synthase isoenzymes whose number ranges from one in *Schizosaccharomyces pombe* to seven in some filamentous fungi, such as *Aspergillus fumigatus*. Chitin synthases are membrane proteins that catalyze the polymerization of N-acetylglucosamine. To date, many chitin synthase genes have been identified from various fungi, and the polypeptides deduced from these genes have been divided into six classes. This high diversity makes it difficult to find a unique model of regulation. However, the results available suggest common themes in regulation. The arrival of the genomic era, together with the development of fungal genetic technology should allow experimental approaches to understand this process (Roncero, 2002).

Chitin synthesis has been studied in several biological systems. UDPGlcNAc is synthesized from fructose 6-phosphate and glutamine by four enzyme catalyzed reactions. The first step of the pathway is the formation of glucosamine 6-phosphate from fructose 6-phosphate and glutamine, a reaction

catalysed by amidotransferase. This enzyme activity was first described by Leloir and Cardini (1953) in *Neurospora crassa*. Once formed glucosamine 6-phosphate undergoes acetylation to N-acetylglucosamine 6-phosphate by the enzyme glucosamine 6-phosphate acetyltransferase. The enzymes for the next two reactions, phospho-N-acetyl glucosamine mutase and N-acetylglucosamine 1-phosphate uridyltransferase, were first identified in *Neurospora crassa* and yeast, respectively. Amidotransferase is the first rate limiting enzymes of the hexosamine biosynthetic pathway. It controls the flux of glucose into hexosamine pathway and thus the formation of hexosamine products and most likely regulates the availability of precursor for chitin synthesis. It also supplies amino sugars for N- and O-linked glycosylation of proteins (Maia, 1994).

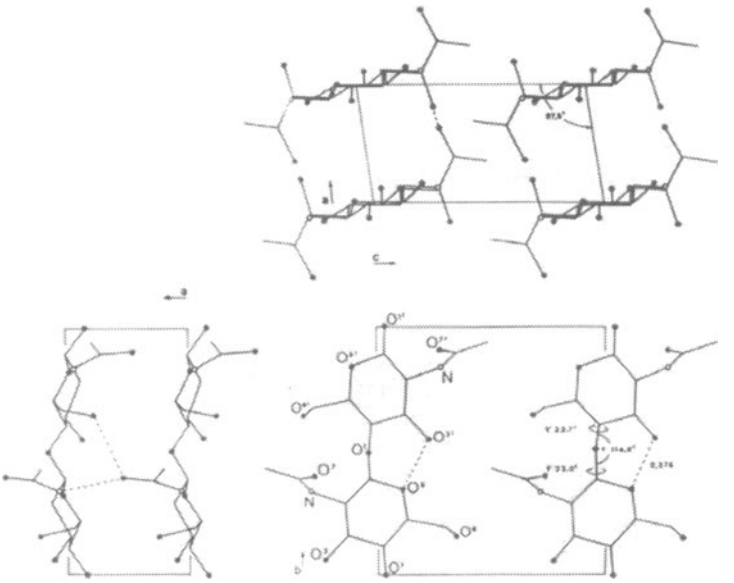

Fig. 15.2 3D-conformation of α-chitin (Ramakrishnan and Prasad, 1972)

Glaser and Brown (1957) reported synthesis of insoluble chitin directly from UDP-*N*-acetylglucosamine (UDPGlcNAc) as the activated sugar donor and the reaction proceeds by glycosyl transfer from nucleotide linked sugar to chitodextrin chains to form the chitin polymer. In *Saccharomyces cerevisiae*, chitin is synthesized by a family of 3 specialized but interacting chitin synthases encoded by CHS1, CHS2 and CHS3. Chs2p makes chitin in the primary septum, while Chs3p makes chitin in the lateral cell wall and in the bud neck (Lesage *et al.*, 2005).

Chitin synthase can be assayed readily and some progress has been made in purifying active components in fungal systems (Machida and Saito, 1993; Uchida *et al.*, 1996). However, despite all efforts that have been made during the past

decades, the enzyme has still not been purified to homogeneity. Therefore, we have only a vague image of the molecular mechanism of chitin synthesis.

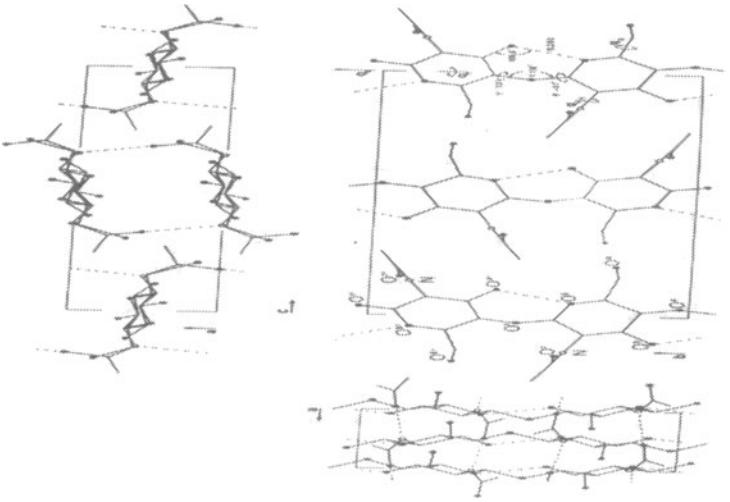

Fig. 15.3 3D-conformation of β-chitin (Gardner and Blackwell, 1975)

Chitin degradation

Chitin synthases and chitinolytic enzymes work hand in hand in remodeling chitinous structures. So far, we have discussed those enzymes that are relevant for chitin synthesis. The degrading enzymes include the chitinases. Chitinases are produced by different micro-organisms which generally present a wide multiplicity of enzymes (Dahiya *et al.*, 2005). Chitinases are the most important enzymes involved in maintaining cell wall structure in fungi. Since chitin is hard to break due to its physicochemical properties, its degradation usually requires the action of more than one enzyme type. Most of the fungal chitinases characterized to date have exochitinase, endochitinase or N-acetyl hexosaminidase activity and a number of these enzymes also exhibit transglycosylase activity. They may therefore contribute to breakage and reforming of bonds within and between polymers, leading to re-modeling of the cell wall during growth and morphogenesis.

Endosplitting chitinases produce chitooligomers that are subsequently converted to monomers by exo-splitting ß-*N*-acetylglucosaminidases. The latter enzyme cleaves off *N*-acetylglucosamine units from non-reducing ends and prefers smaller substrates than chitinases (Filho *et al.*, 2002). As a consequence of these properties, the overall rate of chitin hydrolysis is limited by the action of the chitooligomer-producing chitinase, which drastically increases the effective substrate concentration for the ß-*N*-acetylglucosaminidase. All of them catalyze the hydrolysis of ß-(1-4)-glycosidic bonds of chitin polymers and oligomers.

Since chitin-degrading enzymes can be used to convert chitin-containing raw material into biotechnologically utilizable components, they are of significant interest for the chemical and pharmaceutical industries. Moreover, chitinases and their inhibitors may be adopted as insecticides to combat pests or as fungicides for the treatment of microbial infections (Herrera-Estrella and Chet, 1999).

Chitinase families

Fungal chitinases belong to family 18 of the glycosyl hydrolase superfamily which includes chitinases from bacteria, fungi, plants, insects, mammals, and viruses. All family 18 proteins have an $(\alpha/ß)_8$-barrel fold, where the substrate binding cleft is formed by loops positioned between the carboxyl-terminal end of the ß-strands and the amino-terminal end of the helices (Henrissat and Davies, 1997). The $(\alpha/ß)_8$ topology is found in several families of glycosyl hydrolases with very different substrate specificity and a very low overall sequence homology. The catalytic residues have been identified in a number of these enzymes, and the presence of conserved unusual nonproline cis-peptide bonds provides further evidence for an evolutionary relation between $(\alpha/ß)_8$, or TIM-barrel, glycosyl hydrolases (Herzberg and Moult, 1991). It has been speculated that artificial enzymes could be designed and engineered on the basis of this fold, and recently it was demonstrated that the $(\alpha/ß)_8$ barrel fold is indeed a good scaffold for changing the substrate specificity of a synthase into isomerase by directed evolution (Altamirano *et al.*, 2000).

The family 18 chitinases are supposed to cleave the glycosidic linkage of the chitooligosaccahride (between subsites +1 and −1) with retention of the configuration. It has also been suggested that chitinases apply a substrate-assisted catalysis mechanism involving the catalytic acid and the carbonyl oxygen atom of the substrate's N-acetyl group at subsite −1 as the nucleophile (Tews *et al.*, 1997; Brameld *et al.*, 1998; van Aalten *et al.*, 2001). According to this hydrolysis mechanism, NAG is always needed to occupy the −1 site for the catalysis to occur. The hydrolysis results with different MeUmb-saccharides support this reaction mechanism for both *Trichoderma* chitinases, because only a NAG unit was accepted at subsite −1. PbCTS1 from *Paracoccidioides brasiliensis* is composed of two signature family 18 catalytic domains and seems to belong to fungal/bacterial class (Bonfim *et al.*, 2006).

Phylogenetic analysis of *H. jecorina* chitinases, and those from other filamentous fungi, including hypothetical proteins of annotated fungal genome databases, showed that the fungal chitinases can be divided into three groups: groups A and B (corresponding to class V and III chitinases, respectively) also contained the *Trichoderma* chitinases identified to date, whereas a novel group C comprises high molecular weight chitinases that have a domain structure similar to *Kluyveromyces lactis* killer toxins. Five chitinase genes, representing members of groups A-C, were cloned from the mycoparasitic species *H. atroviridis* (anamorph: *T. atroviride*) (Seidl *et al.*, 2005). Such structural differences

between plant and microbial family 18 chitinases might result in differences in their functional properties, such as substrate-binding mode.

Boer *et al.* (2004) reported two glycosyl hydrolase family 18 chitinases, Chit33 and Chit42, from the filamentous fungus *Trichoderma harzianum* and characterizaed using a panel of different soluble chitinous substrates and inhibitors. They reported substrate-assisted catalysis mechanism for both chitinases. Both *T. harzianum* chitinases are able to catalyze some transglycosylation reactions and cleave both simple chito-oligosaccharides and synthetically modified, beta-1,4-galactosylated and alpha-1,3-fucosylated chito-oligosaccharides.

Fungal chitinases

A number of chitinases have been reported from fungi. Sakurda *et al.* (1996) purified a 42kDa chitinase from *Piromyces communis* OTS1. The pH and temperature optima of enzyme were 4.0-4.5 and 40°C, respectively. It was inhibited by Ag^+, Hg^+ and allosamidin at 1mM concentration. Pinto *et al.* (1997) purified chitinase from *Metarhizium anisopliae*. The purified chitinase had a molecular weight of 30kDa and was optically active at pH 4.5-5.0 and temperature 40-45°C, respectively. A 43kDa chitinase was purified from *Trichoderma harzianum* Rifai T24. Chitinase was stable at 30°C. Its half life at 60°C was 15min (El-Katatny *et al.*, 2001).

Two chitinases P-1 and P-2 were purified from *Isaria japonica*. The molecular weights of enzymes P1 and P2 were 43.273kDa and 31.134kDa, respectively. The optimum pH and temperature were 3.5-4.0 and 50°C for P-1 and 4.0-4.5 and 40°C for P-2. The products from chitin hexamer obtained with P-1 were almost all dimers with only small amount of trimer whereas those with P-2 were mainly trimers with some dimer and tetramer (Kawachi *et al.*, 2001).

Souza *et al.* (2003) purified 43kDa endochitinase from *Colletotrichum gloeosporioides*. The pH and temperature optima were 7.0 and 50°C, respectively. Two isozymes I and II (molecular weight 67kDa) of N-acetyl β-D-glucosaminidases were purified from *Fusarium oxysporum* F3. The optimum pH of isozymes I and II were 5.0 and 6.0, respectively whereas maximum activity of both the isozymes was obtained at 40°C (Gkargkas *et al.*, 2003).

Regulation of fungal chitinase genes

Chitinse gene expression has been reported to be controlled by a repressor/inducer system in which chitin or other products of degradation act as inducers. Vasseur *et al.* (1990) found that chitinase gene expression in a chitinase overexpressing mutant *A. album* E3 was induced by N-acetyl glucosamine while it was repressed by glucose. On the other hand, in gene regulation of *T. harzianum* high chitinase activity was found only in cultures supplied with chitin but not with cellulose, chitosan or chitobiose. In *T. harzianum* N-

acetylglucosamine did not enhance the chitinase production but instead repressed its synthesis (Ulhoa and Peberdy, 1991).

Chit 33 endochitinase of *Trichoderma harzianum* CECT2413 is induced either by carbon or nitrogen source starvation or to a low degree also under conditions of temperature stress (Mercedes *et al.*, 2001). Viterbo *et al.* (2002) reported induction of *chit36Y* (coding for CHIT36) from *Trichoderma harzianum* by stress conditions, colloidal chitin and N-acetyl D-glucosamine. Repression of chitinase synthesis in almost all organisms indicates that catabolite repression may be involved in the regulation of microbial chitinase genes. An endochitinase of *Aspergillus fumigatus* NCPF2140 was induced by chitin (as sole carbon source) and repressed by GlcNAc indicating regulation by a negative feed back mechanism (Escott *et al.*, 1998). A chitinase gene *chi3* encoding CHIT30 from *Metarhizium anisoplae* is upregulated by chitin, tick culture and low concentrations of N-acetylglucosamine (0.25%) and is down regulated by high N-acetylglucosamine (1.0%) and glucose (1%) concentrations (da Silva *et al.*, 2005).

Cloning of fungal chitinase genes

There are several reports on cloning of fungal chitinase gene(s). Pishko *et al.* (1995) reported cloning and sequencing of two chitinase (CTS)-encoding genes (*cts*) from *Coccidioides immitis* (Ci), a respiratory fungal pathogen of humans. They reported that *cts1* gene contains five introns and a 1281-bp ORF which translates a 427-amino-acid (aa) protein of 47.4 kDa and *cts2* gene contains two introns and a 2580-bp ORF which translates a 860-aa protein of 91.4 kDa. The deduced CTS1 protein showed highest homology to the *Aphanocladium album* and *Trichoderma harzianum* CTS (74% and 76%, respectively), while CTS2 showed highest homology to the CTS of *Saccharomyces cerevisiae* (Sc) and *Candida albicans* (47% and 51%, respectively). Takaya *et al.* (1998) reported cloning of chitinase-encoding gene from *Aspergillus nidulans* by polymerase chain reaction using degenerated oligonucleotide primers designed from the conserved amino acid sequences among chitinases from yeasts and *Rhizopus* spp. The cloned gene, named *chiA*, encoded a polypeptide consisting of 660 amino acids. They studied expression of chiA gene using *Escherichia coli* beta-galactosidase as a reporter enzyme. Using *in situ* staining they found high expression of beta-galactosidase in metulae, phialides, and conidia during conidiophore development, indicating developmentally regulated expression of chiA. Takaya *et al.* (1998) reported the primary structure of an intracellular chitinase (chitinase III) from *Rhizopus oligosporus*, a zygomycete filamentous fungus. The gene encoding chitinase III (*chi3*) was cloned using PCR with degenerate oligonucleotide primers from the partial amino acid sequence of the enzyme. The deduced amino acid sequence of Chi3 was similar to that of bacterial chitinases and chitinases from mycoparasitic fungi, such as *Aphanocladium album* and *Trichoderma harzianum*, but it had no potential secretory signal sequence in its amino terminus. Northern blot analysis

showed that *chi3* was transcribed during hyphal growth. These results suggest that chitinase III may function during morphogenesis in *R. oligosporus*.

Boga *et al.* (1998) reported the complete nucleotide sequence and analysis of the chromosomal and full-length cDNA copies of the regulated gene (chit1) coding one of the chitinases produced by the biocontrol agent *Metarhizium anisopliae*. Albeit at least two different chitinases are characterized in this fungus, only one chitinase gene was isolated. The chit1 gene is interrupted by three short typical fungal introns and has a 1,521-bp ORF, which encodes a protein of 423 amino acids with a stretch of 35 amino acid residues displaying characteristics of signal peptide.

Kim *et al.* (2002a) reported the characterization of a diverse set of chitinase genes from *T. virens*. The full-length genomic clones were isolated and characterized, while sequencing of the corresponding cDNA clones and manual annotation provided a basis for establishing gene structure. Based on homology of the deduced amino acid sequences, they identified three members of the 42Kd endochitinase gene family, two 33Kd exochitinases and two exochitinases with homology to N-acetylglucosaminidases. Expression of the endochitinase encoding *ech42* gene of the mycoparasite *Trichoderma atroviride* is reported to be derepressed by carbon starvation (Brunner *et al.*, 2003). For identifying promoter areas involved in control by this condition, they prepared fusions of several mutant forms of the *ech42* promoter to the *Aspergillus niger* goxA gene as a reporter. Using protein-DNA binding analyses they reported that only the BrlA-like sites, but neither the AGGGG element nor the Cre1 binding site, bound proteins from cell-free extracts from carbon-starved mycelia of *T. atroviride* thus they identified a new regulator of chitinase gene expression in *Trichoderma*.

A chitinase gene encoding an inducible 45 kDa chitinase of *Aspergillus fumigatus* was cloned by Jaques *et al.* (2003). Based on deduced amino acid sequence, it was identified as a chitinase of the fungal/bacterial class, which was, designated ChiB1. Recombinant ChiB1, expressed in *Pichia pastoris*, was shown to function by a retaining mechanism of action. Fang *et al.* (2005) cloned a chitinase gene coding for an endochitinase (Bbchit1) from *Beauveria bassiana*. They reported that *Bbchit1* was intronless, and there was a single copy in *B. bassiana*. Its regulatory sequence contained putative CreA/Crel carbon catabolic repressor binding domains, which was consistent with glucose suppression of Bbchit1. At the amino acid level, Bbchit1 showed significant similarity to a *Streptomyces avermitilis* putative endochitinase, a *Streptomyces coelicolor* putative chitinase, and *Trichoderma harzianum* endochitinase Chit36Y.

Inhibition of chitin metabolism

Chitin synthesis can be blocked during the various steps by a variety of antibiotics, metabolic inhibitors, insect growth regulators, alkaloids and hormone analogs. Chitin synthesis inhibitors as well as inhibitors of chitin degradation that

produce similar effects are promising agents for controlling insect pests, fungal pathogens and helminthic parasites.

Inhibitors of chitin synthase

Inhibitors of chitin synthesis have been classified into three major groups: peptidyl nucleosides, acyl ureas and substances interfering with hormonal control. Peptidyl nucleosides isolated from diverse *Streptomyces* species act as substrate analogues and include polyoxins and nikkomycins (Zhang and Miller, 1999). They competitively inhibit both fungal and insect chitin synthases. Although it is well established that acyl ureas such as diflubenzuron and teflubenzuron affect chitin synthesis (van Eck, 1979), their mode of action is still puzzling. However, several lines of experiment argue against a direct interaction of these inhibitors with the chitin synthase. For instance, in cell-free systems, acyl urease do not inhibit chitin synthesis (Mayer *et al.*, 1980). Instead of directly blocking chitin synthase activity, they may alter either vesicle transport or fusion, inhibit the translocation of chitin fibrils across the plasma membrane (Cohen, 2001) or interfere with the hormonal regulation of chitin synthesis by influencing ecdysteroid production (Fournet *et al.*, 1995).

The third group of inhibitors evidently affects hormonal regulation of insect growth and development. One of the manifold effects of these substances is certainly deregulation of chitin synthesis, probably by preventing the expression of the chitin synthase or regulating factors. Djebaili and Behr (2005), identified 6-deoxy-homoDMDP as a potent inhibitor of chitin synthase (Ki = 38 microM), displaying an uncompetitive inhibition pattern. Phellinsin A, a novel chitin synthases inhibitor was isolated from the cultured broth of fungus PL3, which was identified as *Phellinus* sp. PL3 (Hwang *et al.*, 2000). Obovatol inhibited chitin synthase 2 activity of *Saccharomyces cerevisiae* with an IC_{50} of 38 μM. Its derivative, tetrahydroobovatol, inhibited chitin synthase 2 activity under the same conditions with an IC_{50} of 59 μM (Hwang *et al.*, 2002). Nikkomycin Z is a competitive inhibitor of chitin synthases in fungi. The IC_{50} value for *Candida albicans* (CaChs1) is 15μM, for CaChs2 is 0.8μM, and for CaChs3 is 13μM (Kim *et al.*, 2002b). 3-O-galloyl-(-)-shikimic acid was the most potent inhibitor against chitin synthase II of *Saccharomyces cerevisiae* with an IC_{50} value of 18μM (Hwang *et al.* 2001).

Ursolic acid inhibits chitin synthase II from *S. cerevisiae* with an IC_{50} value of 0.84μg/ml. Oleanolic acid, alpha-hederin hydrate, and betulic acid inhibited the chitin synthase II activity under the same conditions with an IC_{50} of 5.6, 64.3, and 98.7μg/ml, respectively (Jeong *et al.*, 1999). Two flavonoids, (+/-)-catechin and (-)-epicatechin inhibit chitin synthase II with an IC_{50} of 15 and 29 μg/ml, respectively (Kim *et al.*, 1999). A novel chitin synthase inhibitor that is highly specific to CaChs1p designated RO-09-3143 inhibited the septum formation and the growth of *C. albicans* cells (Sudoh *et al.*, 2000).

Inhibitors of chitinase

In addition to general enzyme inhibitors, such as organic compounds and oxidizing/reducing agents a number of reports are available on the natural chitinase inhibitors. Chitinase inhibitors can generally be grouped into two major classes: they mimic either carbohydrate substrates or the oxocarbonium reaction intermediate of family 18 chitinases.

The first chitinase inhibitor, allosamidin, a pseudotrisaccharide was isolated from the mycelial extract of *Streptomyces* sp. no. 1713 (Sakuda *et al.*, 1987). It is similar to N-acetyl D-glucosamine but lack a pyranose ring-oxygen and contains an oxazoline ring in which the methyl group is substituted by dimethylamine (Fig. 15.4). Allosamidin exerts its inhibitory effect by acting as a nonhydrolyzable analogue of the oxazolinium ion inermediate (Tews *et al.*, 1997). Allosamidin is a potent chitinase inhibitor; however, its production is expensive because it is difficult to synthesize.

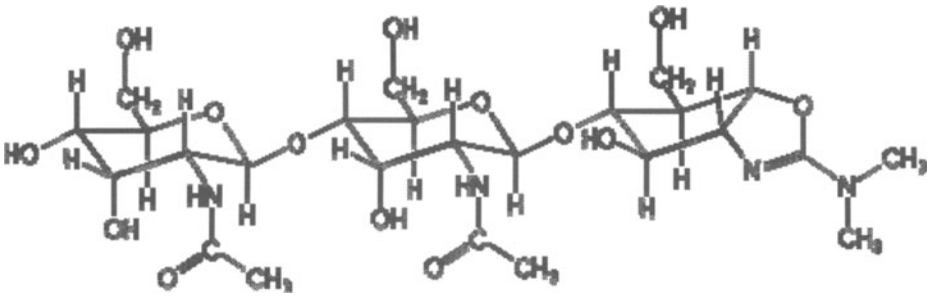

Fig. 15.4 Structure of allosamidin

In the course of the screening for new insecticides, two new cyclopentapeptide chitinase inhibitors, argifin (Omura *et al.*, 2000) and argadin (Arai *et al.*, 2000), were found from the fungal cultures of *Gliocladium* sp. FTD-0668 and *Clonostachys* sp. FO-7314, respectively. These molecules are as potent inhibitors as allosamidin but synthesis by peptide chemistry is less expensive. Both argifin and argadin interact with side chains (Asp-142, Glu-144, and Tyr-214) in the chitinase active site that are conserved completely and required for catalytic activity in family 18 chitinases (van Aalten *et al.*, 2001).

While the cyclopentapeptides are carbohydrate mimics, the small peptide CI-4, which was recently identified in the marine bacterium *Pseudomonas*, functions like allosamidin as a mimic of the family 18 chitinases' catalytic transient state (Houston *et al.*, 2002). Recently, a high throughput screen identified three xanthine derivatives, theophylline, caffeine and pentoxifylline, as competitive inhibitors of bacterial, fungal and human family 18 chitinases (Rao *et al.*, 2005). Vaaje Kolstad *et al.* (2004) designed a new chitinase inhibitor N,N'-diacetylchitobionoxime-N-phenylcarbamate (HM508) which acts as a

competitive inhibitor of this enzyme with a K(i) in the 50 microM range against *Serratia marcescens* chitinase (ChiB).

Outlook

Chitin is an important component of fungal cell wall. Chitin metabolising enzymes have important biological and physiological roles containing autolytic, nutritional, morphogenetic and parasitic roles. As chitin synthesis and degradation are crucial for growth and development of fungi and are thus ideal targets for disruption and perturbation by novel fungicides. The review has focused on the recent advances made in the field of chitin metabolism in fungi. Chitin synthesis, transport and deposition are complex processes in which chitin chains are synthesized, assembled as microfibrils and cross linked with other cell wall components. Despite all new insights resulting from cloning and sequencing, however, we are still far away from understanding the detailed modes of action of the enzymes involved, especially chitin synthases and chitinases. Moreover, we do not actually know the regulatory mechanisms that control and coordinate enzyme biosynthesis and activity during development. Thus, the regulatory mechanism that control chitin synthase activity need to be analyzed in greater detail. Although biocontrol of pathogenic fungi using chitinases has been studied, the future research should be on discovery of novel chitinases, construction of genetically engineered chitinases and study of its regulatory mechanism. The information may prove more useful in design of novel antifungal agents for controlling pathogenic fungi.

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CHAPTER - 16

FUNGI-MEDIATED SYNTHESIS OF SILVER NANOPARTICLES: CHARACTERIZATION PROCESSES AND APPLICATIONS

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Introduction

The term "nanotechnology" is derived from the Greek word 'nano', meaning 'dwarf', and applies to the principles of engineering and manufacturing at a molecular level. The common definition of nanotechnology is that of manipulation, observation, measurement and synthesis at a scale of 1 to 100 nanometers (Raj and Asha, 2009). Nanobiotechnology is a new branch of science dedicated to the improvement and utilization of devices and structures ranging from 1 to 100 nm in size, in which new chemical, physical, and biological properties, not seen in bulk materials, can be observed. There is tremendous excitement in this field with respect to their fundamental properties, organization of superstructure and applications.

Nanomaterials exhibit a number of special properties; therefore, it may provide solutions to technological and environmental challenges in different areas like medicine, agriculture, solar energy conversions, catalysis and water treatment (Dahl *et al.*, 2007; Hutchison, 2008). Nanoparticles have a very high surface area to volume ratio, this provides a tremendous driving force for diffusion, especially at elevated temperatures and hence it plays an important role in field of drug delivery (Mah *et al.*, 2000).

The synthesis of metallic nanoparticles followed by a green-procedure is of great interest in the actual environmental concern. There are many production processes of silver nanoparticles but the biological one is playing an important role for avoiding the environmental pollution caused by physical and chemical procedures. Many reviews on the biosynthesis of metallic nanoparticles were already published showing the importance of these nanoparticles (Sastry *et al.*, 2003; Senapati *et al.*, 2004; Mandal *et al.*, 2006; Mohanpuria *et al.*, 2008; Bhattacharya and Mukherjee, 2008; Chen and Schluesener, 2008; Sharma *et al.*, 2009; Rai *et al.*, 2009).

Many fungi have been used to produce silver nanoparticles intracellularly or extracellularly. Several fungi were studied, such *as Verticillium* (Mukherjee *et al.*, 2001a), *Phoma sp* (Chen *et al.*, 2003; Birla *et al.*, 2009), *Phaenerochaete chrysosporium* (Vigneshwaran *et al.*, 2006), *Aspergillus niger* (Gade *et al.*, 2008), *Aspergillus fumigatus* (Bhainsa and D'Souza, 2006), *Aspergillus flavus* (Vigneshwaran *et al.*, 2007), *Fusarium oxysporum* (Ahmad *et al.*, 2003; Senapati *et al.*, 2004; Durán *et al.*, 2005; 2007), *Fusarium semitectum* (Basavaraja *et al.*, 2008), *Fusarium acuminatum* (Ingle *et al.*, 2008), *Fusarium solani* (Ingle *et al.*, 2009), *Penicillium* (Sadowski *et al.*, 2008), *Trichoderma asperellum* (Mukherjee *et al.*, 2008), *Coriolus versicolor* (Sanghi and Verma, 2009) and *Cladosporium cladosporioides* (Balaji *et al.*, 2009).

The present chapter focuses on various methods required for the characterization of silver nanoparticles, fungal species used for the synthesis of metal nanoparticles and application of nanoparticles in various fields including opto-electronics, medicine, biosensors, DNA labeling, etc.

Characterization

The silver nanoparticles can be characterized by different techniques such as Ultraviolet-Visible spectroscopy, fluorescence, fourier-transformed-infrared spectroscopy, X-ray diffraction, microscopy techniques, and photoluminescence spectra. These techniques can provide the information about the formation, size and morphology of the particles, capping, stability and other information as described in more detail below.

Ultraviolet-Visible Spectroscopy

The formation and stability of silver nanoparticles can be monitored by UV–Vis spectral analysis. Metallic nanoparticles smaller than or comparable to the penetration depth of electromagnetic fields in the metal have surface plasmon resonance. This phenomenon causes a strong absorption and scattering of visible and infrared light that explain the color of colloidal solutions of different metals (Krasovskii and Krasovskii, 2008). In the specific case of silver nanoparticles, the band in the UV-Vis spectrum corresponding to the surface plasmon resonance occurs at around 415-470 nm.

The plasmon absorption can be used to verify the formation of silver nanoparticles. The shape and position of plasmon resonance depends on a number of factors such as the dielectric constant of the medium, size and shape of the particles, surface-adsorbed species, etc. The Fig. 16.1 shows the UV-Vis spectrum of silver nanoparticles produced by *Fusarium oxysporum*. An increase in the plasmon absorption (ca. 420 nm) can be observed with time indicating the formation of silver nanoparticles formation. The maximum wavelength can vary with the size and the shape of silver nanoparticles. The maximum absorption is dislocated to larger wavelength with the decrease of particle size (Vigneshwaran *et al.*, 2006).

Silver nanoparticles with different size and shape can be produced by biological method depending on concentration of the Ag^+ ion in solution, the enzymes released by fungal strains and pH of the solution. The size and shape of particles are important factors in application of silver nanoparticles (He *et al.*, 2002; Balaji *et al.*, 2009).

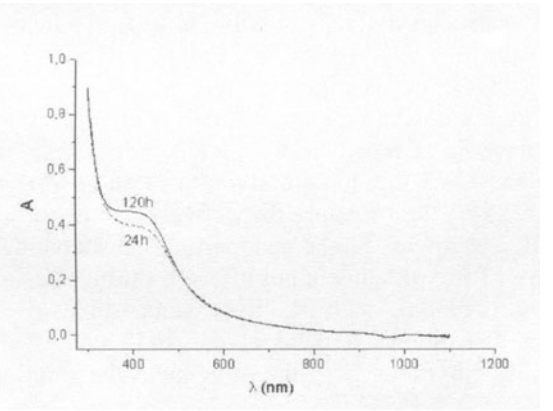

Fig. 16.1: UV-Vis spectra as a function of time of reaction of an aqueous solution of 10^{-3} M $AgNO_3$ with the fungal filtrate from *Fusarium oxysporum*.

Other important absorption band to silver nanoparticles is at 378 nm. This band appears as shoulder and corresponds to the transverse plasmon vibration in the silver nanoparticles while the peak at 420 nm is due to excitation of longitudinal plasmon vibrations. This band as well as the band around 420 nm indicates the formation of silver nanoparticles. The separation between the bands indicate that silver nanoparticles are formed mostly as aggregates (Basavaraja *et al.*, 2008).

The UV-Vis spectroscopy can be used to characterize the protein around the biosynthesized silver nanoparticles. The aromatic amino acids of protein present an absorption band at ca. 270 nm. This band is due to electronic excitations in tryptophan and tyrosine residues in the proteins. The interaction between protein and silver nanoparticles can also be studied by fluorescence and fourier-

transformed-infrared spectroscopy as described below (Ahmad *et al.*, 2003; Durán *et al.*, 2005).

Fluorescence

The fluorescence technique can characterize the interactions of protein-nanoparticle. When silver nanoparticles dispersion is excited in λ = 260 nm (maximum optical transitions in tryptophan and tyrosine residues of proteins), a band centered at ca. 340 nm appears. The nature of this emission band indicates that the proteins bound to the surface of nanoparticles and those present in the solution in the native form. Probably in the reduction process the tertiary structure of the proteins is not affected with the binding of silver nanoparticles to the surface (Ahmad *et al.*, 2003; Durán *et al.*, 2005).

Fourier-Transformed-Infrared spectroscopy (FTIR)

The FTIR spectroscopy is very important to characterize the protein binding with the silver nanoparticles and it is possible to quantify secondary structure in metal nanoparticle–protein interaction. The position of bands in the FTIR spectra from the amide I and II of proteins are sensitive indicator of conformational changes in the protein secondary structure (Gole *et al.*, 2001). The band in FTIR of amide I and amide II of native protein appears at around 1640 and around 1540 cm^{-1}. These bands are due to carbonyl stretch and –N–H stretch vibrations in the amide linkages of the proteins, respectively. Thus, the presence of these bands in the FTIR spectra of silver nanoparticles dispersion indicate that the secondary structure of the proteins is not affected during the formation of silver nanoparticles or by its binding with the silver nanoparticles (Balaji *et al.*, 2009). The FTIR spectra also shows a band at ca. 1450 cm^{-1} that corresponds to methylene scissoring vibrations from the proteins in the solution (Ahmad *et al.*, 2003).

FTIR also supply information about thiol derivatives surrounding the metallic cores giving more details about the protein capping to the silver nanoparticles. The –SH stretches in native protein occur at 2590–2540 cm^{-1}. The absence of the band in this region indicates the formation of a bond between the S atoms and silver clusters (chemisorption) (Tan *et al.*, 2002; Sanghi and Verma, 2009).

X-ray diffraction (XRD)

XRD is an important technique to evaluate the formation of silver nanoparticles and to determine the particle size. Silver nanoparticles produced by fungi showing a number of Bragg reflections corresponding to the (1 1 1), (2 0 0), (2 2 0) and (3 1 1) sets of lattice planes. These planes correspond to FCC structures of silver (index JCPDS file no. 03-0921). This result demonstrate crystalline nature of silver nanoparticles.

The size of silver nanoparticles can be calculated by Debye-Scherrer's equation:

$$D = (K\lambda)/ (\beta_{cor} \cos\theta), \text{ with } \beta_{cor} = (\beta^2_{sample} - \beta^2_{ref})^{1/2}$$

Where D is the average crystal size, K is the Scherrer's coefficient (0.89), λ is the X-ray wavelength (λ = 1.542 Å), θ Bragg's angle (2θ = 25.1°), β_{cor} the corrected of the full width at half-maximum (FWHM) in radians, β_{sample} and β_{ref} are the FWHM of the reference and sample peaks, respectively (Durán *et al.*, 2007). The diffraction signal in (111) plane of 2θ spectrum is used to calculate the silver nanoparticle size by Scherrer's equation.

In general, the calculated average particle size of the silver by XRD is also in line with the observation of the TEM results (Basavaraja *et al.*, 2008; Balaji *et al.*, 2009).

Transmission Electron Microscopy (TEM)

Morphology of silver nanoparticles can be observed by microscopic techniques such as TEM and SEM. TEM micrographs of nanoparticles can show whether the silver nanoparticles are symmetrical and with spherical shaped, whether the particles form aggregate or not and also determine the average size. Furthermore, it is possible to do the elemental analysis using the Elemental Spectroscopy Imaging (ESI). This analysis is very important to silver nanoparticles produced by microorganisms because it can characterize the stabilization by capping around the particles to confirm if fungal proteins are responsible for covering and stabilizing the particles or not (Vigneshwaran *et al.*, 2006; Duran *et al.*, 2007; Sanghi and Verma, 2009). TEM images can also be used to localize the silver nanoparticles in fungal or bacterial cell and this could be important to elucidate the mechanism of their formation or their antimicrobial activity, respectively (Mukherjee *et al.*, 2001b).

TEM under high resolution of a silver nanoparticles sample can analyze the nanoparticles preservation for long time. This led to observe the effect of ageing on the size of the nanoparticles. Furthermore, mean value of particle size can be estimated from the particle size histogram assuming a log-normal distribution of the particle diameter D according to the equation given below:

$$f(D) = (1/D\sigma\sqrt{2\pi}) \text{ [exp] } \{-[\ln(D/D_0)]^2/ 2\sigma^2\}$$

Where D_0 (15 nm) is the estimated median value for particle diameter and σ (0.1) is the distribution width (Mukherjee *et al.*, 2008).

Scanning Electronic Microscopy (SEM)

Morphology of silver nanoparticles and their elemental characterization can be made by SEM combined with Energy Dispersive X-ray Spectroscopy (EDS). The sample needs to be dried before its analysis by SEM because this technique is made under vacuum. But, high magnifications in ''wet mode'' can be made by

Environmental Scanning Electron Microscope (ESEM). This technique can be associated with a specially designed cooling stage, combined with energy diffraction analysis of X-rays (EDX) are used in some cases in silver nanoparticles determination in fungal cells. It is possible to be observed by ESEM silver nanoparticles on the surface of the mycelial mat when silver nanoparticles are formed in presence of fungal mycelium. The EDX coupled to ESEM allows the elemental analysis of isolated nanoparticles and particles on surface of fungal mycelium confirming the presence of these nanoparticles in the mycelium (Vigneshwaran *et al.*, 2006). Silver location of nanoparticles and their distribution on surface of the fungal cell can also be observed by SEM (Mukherjee *et al.*, 2001b; Vigneshwaran *et al.*, 2007).

SEM micrograph of silver nanoparticles shows, in many cases, aggregated particles due to the capping agent. Therefore, the particles size measured by SEM can be larger than the size measured by TEM or XRD (Durán *et al.*, 2005).

Photoluminescence

Photoluminescence is an effective method to evaluate the optical property of metallic nanoparticles as photonic materials. Silver nitrate solution does not show emission peak but the silver nanoparticles produced by fungus *Phanerochaete chrysosporium* showed an emission peak at 423 nm with very broad base. This result can be explained by the ability of silver nanoparticles to enhance the fluorescence emission intensity and photostability of nearby fluorophores. Therefore, the emission peak intensity at 423 nm of protein bound to silver nanoparticles might be increased (Vigneshwaran *et al.*, 2006). Curiously, the emission peak of silver nanoparticles produced by *Aspergillus flavus* was at 553 nm with very broad base, and no explanation for this big difference was provided (Vigneshwaran *et al.*, 2007).

Characterization of silver nanoparticles synthesized by fungi

Verticillium

Verticillium is a genus of fungi in the division Ascomycota. Within the genus, diverse groups are formed comprising saprophytes and parasites of higher plants, insects, nematodes and other fungi, thus it can be seen that the genus is a wide ranging group of taxa characterised by simple but ill-defined characters. The genus may be broadly divided into three ecologically based groups, (i) mycopathogens (ii) entomopathogens (Zare and Gams, 2001), and (iii) plant pathogens and related saprotrophs (Barbara and Clewes, 2003).

Recently, the genus has undergone some revision into which most entomopathogenic and mycopathogenic isolates fall into a new group called *Lecanicillium*. Plant pathogenic isolates still retain the original genus name *Verticillium*. The better known species of *Verticillium* are *V. dahliae* and *V. albo-*

atrum that cause a wilt disease called Verticillium -wilt in more than 300 dicot plant species (Zare and Gams, 2001).

Biotransformation of silver ions by *Verticillium* (Mukherjee *et al.*, 2001a), was monitored by visual inspection of the biomass as well as measurement of the UV-Vis spectra from the fungal cells and the aqueous medium in the reaction mixture. The silver nanoparticles in fungal mycelium were characterized by UV-Vis spectroscopy (Shimadzu dual-beam spectrophotometer-model UV-1601PC), X-ray diffraction (XRD- Leica Stereoscan-440), and scanning electron microscopy (SEM equipped with a Phoenix EDX attachment). The location of the silver nanoparticles in the *Verticillium* cells was investigated by transmission electron microscopy (TEM-JEOL Model 1200EX instrument operated at an accelerating voltage of 60 kV) through the thin sections of *Verticillium* cells after their exposition with Ag^{+} ions .

The UV-Vis spectra recorded from a biofilm of the fungal cells before and after immersion in silver ions showed that the fungal cells exposed to these ions showed absorbance at ca. 450 nm. The presence of the broad resonance peak indicated an aggregated structure of the silver particles in the film. The presence of uniformly distributed silver nanoparticles on the surface of the fungal cells by SEM was observed. The EDX spectrum recorded in the spot profile mode showed densely populated silver nanoparticle regions on the surface of the fungal cells. The TEM image showed small particles of silver organized on the mycelial wall as well as some larger particles within the cells (particle diameter of 25 ± 12 nm).

Phoma

Phoma is a genus of common coelomycetous soil fungi belongs to phylum Ascomycota. It contains many plant pathogenic species. About 200 *Phoma* taxa have been defined and recognized which may be divided into two large groups: (i) plurivorous fungi, generally saprobic or weakly parasitic, mainly from temperate regions in Eurasia, but occasionally also found in other parts of the world (including areas with cool or warm climates); and (ii) specific pathogens of cultivated plants (Van der Aa *et al.*, 1990).

Conidia are colorless and usually unicellular. The pycnidia are black and depressed in the tissues of the host (Rai and Rajak, 1985). *Phoma* is arbitrarily limited to those species in which the conidia are less than 15 μm as the larger conidial forms have been placed in the genus *Macrophoma*. The most important species include *Phoma exigua, P. glomerata, P. sorghina, P. multirostrata, P. medicaginis* and *P. betae*, etc.

Phoma sp. was used as producer of silver nanoparticles for the first time by Chen *et al.* (2003). The quality/quantity adsorption of silver nanoparticles in fungal mycelium was carried out using an atomic absorption spectrometer (AAS) (HITACHI Z-8000, Hitachi, Tokyo, Japan or SOLAAR-M6, Spectronic Unicam Ltd, Rochester, NY, USA). The size of Ag particles adsorbed on the mycelium

surface was observed and measured under TEM (HITACHI H-800). The surface atoms of silver particles on the mycelium were measured by XPS (XSAM800-Kratos, Manchester, UK).

According to the quality and quantity assay by atomic absorption spectrometer (AAS), each gram of dry mycelium produced around 13.4 mg of silver and the amount of Ag adsorbed was 13.9 $mg.L^{-1}$. This value was lower than other fungi like *Aspergillus terreus*. The lower adsorption capacity can be due to freeze-drying process used before the mycelium is exposed to silver ions.

The particles size measured by TEM was 71.06 ± 3.48 nm and this analysis showed a great number of tiny silver particles on mycelium. As per the XPS analyses and according to the standard (Ag_{3d} = 368.2 eV), it could be concluded that Ag^{+} was exactly reduced to Ag^{o}.

Another species of *Phoma* used for the synthesis of silver nanoparticles was *P. glomerata* (Birla *et al.,* 2009). In this study, the authors reported extracellular synthesis of silver nanoparticles when the fungal cell filtrate was treated with aqueous silver ions (silver nitrate 1mM) and incubated at room temperature. The silver nanoparticles synthesized were found to be in the range of 60-80nm when the colloidal solution of these silver nanoparticles was analyzed by using scanning electron microscopy (JEOL 6380A) (Mycelial growth of *P. glomerata* shown in Fig. 16. 2 A).

Further, Birla *et al.* (2009) evaluated the effect of silver nanoparticles in combination with commercially available antibiotics against human pathogenic bacteria viz. *E. coli, Pseudomonas aeruginosa* and *S. aureus* and reported that the antibacterial activities of ampicillin, gentamycin, streptomycin and vancomycin were enhanced in combination with silver nanoparticles against the Gram-negative micro-organisms, i.e. *E. coli* and *P. aeruginosa* as compared with *S. aureus*.

Phaenerochaete chrysosporium

Extracellular biosynthesis of silver nanoparticles by *Phaenerochaete chrysosporium* (a white-rot fungus) was reported by Vigneshwaran *et al.* (2006). The particles were characterized by UV–Vis spectroscopy (Specord 50 Analytikjena® spectrophotometer), X-ray diffraction analysis (Philips® automatic X-ray diffractometer with Philips® PW 1830 X-ray generator), scanning electron microscopy (Philips® XL 30 SEM at 10–17.5 kV), transmission electron microscopy (Philips® EM208 operating at 200 kV) and photoluminescence spectroscopy (Perkin-Elmer LS55® Spectrofluorimeter using 90° illumination). The particles exhibited the plasmon band at 470 nm. The particle size reduction shifts the plasmon band to larger wavelength. Thus, the plasmon band shift from 413 nm to 470 nm and the band was broad with an absorption tail in the longer wavelengths, which could be due to the size and shape distribution of the particles. The same conclusion was afforded with the XRD measurements. Fungal mycelium after challenged with silver ions was

analyzed by ESEM showing silver nanoparticles on the surface of the mycelial mat with size of approximate from 50 to 200 nm. The ESEM images indicate the reduction process being held on the surface. The TEM images showed particles not uniform size and shape with predominance pyramidal shape with different size. The photoluminescence spectra of silver nitrate solution treated with the fungus, showed an emission peak at 423 nm with very broad profile (fungal protein bound to nanoparticles).

Aspergillus niger

Aspergillus niger is one of the most common species of the genus *Aspergillus*. It causes a disease called black-mold on fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* (species of which have also been called "black mold") (Samson *et al.*, 2001).

Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins (Abarca *et al.*, 1994), but other sources disagree, claiming this report is based upon misidentification of the fungal species. Recent evidence suggests that *A. niger* strains do produce ochratoxin A (Samson *et al.*, 2001; Schuster *et al.*, 2002).

Extracellular biosynthesis of silver nanoparticles by *Aspergillus niger* isolated from soil has been reported by Gade *et al.* (2008). The UV-Vis spectra (Perkin Elmer, Labda-25) showed a band centered at 420 nm that increased with the time demonstrating the formation of silver nanoparticles. Furthermore, the color of fungal filtrate changed from dark-yellow to dark-brown which confirms the formation of nanoparticles (Bhainsa and D'Souza, 2006). Homogeny and spherical particles were observed by TEM (Carl Zeiss CEM-902 transmission electron microscope 80 KeV, equipped with a Castaing-Henry-Ottensmeyer energy filter spectrometer within the column). Elemental Spectroscopy Imaging (ESI) was carried out using monochromatic electrons corresponding to the sulfur L2,3-edge. The energy-selecting slit was set at 367 ± 6 keV for Ag and 165 ± 6 eV for S. The images were recorded by a Proscan high-speed slow-scan CCD camera and processed by AnalySis 3.0 system. In this analysis S and N atoms were observed around the silver nanoparticles indicating the association between the nanoparticles and fungal proteins. This protein capping around the particles are responsible for their stabilization by long periods. The protein was also characterized by fluorescence in similar way as previously published, where a fluorescence emission spectra of fungal filtrate exhibited a peak centered at 340 nm (Durán *et al.*, 2005).

TEM images were also used to study the antimicrobial effect of silver nanoparticles. TEM images showed the efficient antimicrobial effect of these particles that disrupted completely the cell membrane after 60 minutes. Furthermore, silver nanoparticles inside the bacteria were observed

demonstrating the capacity of silver nanoparticles to penetrate in to bacterial cell. Mycelial growth of *A. niger* is shown in Fig. 16.2 B.

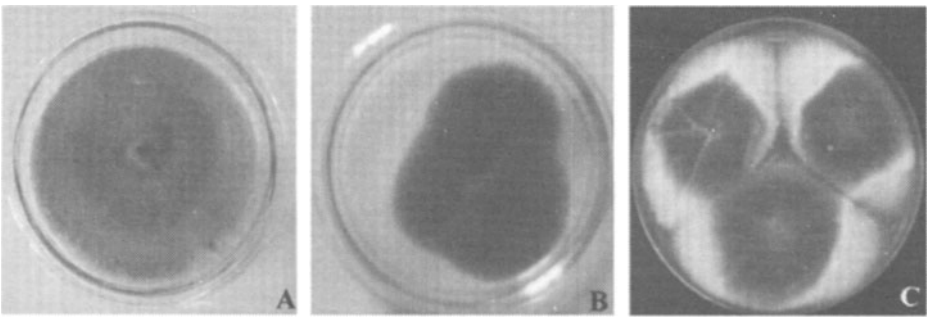

Fig. 16.2. Growth on PDA: (A) *Phoma glomerata* (B) *Aspergillus niger* and (C) *A. fumigatus*

Aspergillus fumigatus

A. fumigatus is a saprotrophic fungus that is widespread in nature, typically found in soil and decaying organic matter such as compost heaps, where it plays an essential role in carbon and nitrogen recycling. Colonies of the fungus produce thousands of minute grey-green conidia (2-3 μm) from conidiophores (Fig.16.2 C). Until recently, *A. fumigatus* was thought to reproduce only by asexual methods, as neither mating nor meiosis had ever been observed in the fungus. However, in 2008 it was shown for the first time that *A. fumigatus* possesses a fully functional sexual reproductive cycle, 145 years after its original description by Fresenius (O'Gorman *et al.*, 2008).

The fungus is capable of growth at 37°C (human body temperature), but can grow at temperatures up to 50°C, with conidia surviving at 70°C. It regularly encounters in self-heating compost heaps. The conidia are ubiquitous in the atmosphere and it is estimated that everybody inhales several hundred spores each day; typically, however, these are quickly eliminated by the immune system in healthy individuals. In immunocompromised individuals such as transplant patients and people with AIDS or leukaemia the fungus is capable of becoming pathogenic and causing a range of diseases generally termed aspergillosis (O'Gorman *et al.*, 2008).

Silver nanoparticles produced by a *Aspergillus fumigatus* (Bhainsa and D'Souza, 2006) were characterized by the absorbance in an UV–Visible spectrophotometer (UV–Vis UV4, UNIVAM Ltd., UK), by TEM (TECNAI 120 at a voltage of 120 kV) and by X-ray diffraction (XRD) using Phillips PW 1710. An increase in the surface plasmon resonance centered at ca. 420 nm was observed until 72 hours of reaction. This results indicated an increased number of particles formed. Nanoparticles with variable shape were observed by TEM. Most particles exhibited spherical morphology and occasionally with triangular shape. The particle-size was in the range 5-25 nm. XRD analysis confirmed the

formation of crystalline particles exhibiting four intense peaks in the whole spectrum of 2θ value. The four intense peaks observed in the spectrum agree to the Braggs's reflection of silver nanocrystals reported in literature

Aspergillus flavus

Aspergillus flavus belongs to Ascomycota (Fig. 16.3A). It is also a common mold in the environment, and can cause storage problems in stored grains. *A. flavus* is particularly common on corn and peanuts, as well as water damaged carpets, and is one of the several species of mold known to produce aflatoxin which cause acute hepatitis, immunosuppression and hepatocellular carcinoma. It can also be a human pathogen, associated with aspergillosis of the lungs and sometimes causing corneal, otomycotic, and nasoorbital infections. Many strains produce significant quantities of aflatoxin (Klich, 2007), a carcinogenic and acutely toxic compound. *A. flavus* spores are allergenic.

Aspergillus flavus is the second most common agent of aspergillosis, the first being *A. fumigatus*. *A flavus* may invade arteries of the lung or brain and cause infarction. Neutropenia predisposes to *Aspergillus* infection. It also produces a toxin (aflatoxin) which is one of the aetiological agents for hepatocellular cacinoma (Crawfold, 2005). It grows as a yellow-green mold in culture. It produces a distinctive conidiophore composed of a long stalk supporting as inflated vesicle. Conidiogenous cells on the vesicle produces conidia. Many strains of *A. flavus* exhibit a greenish fluorescence under UV light that is correlated with levels of aflatoxin production.

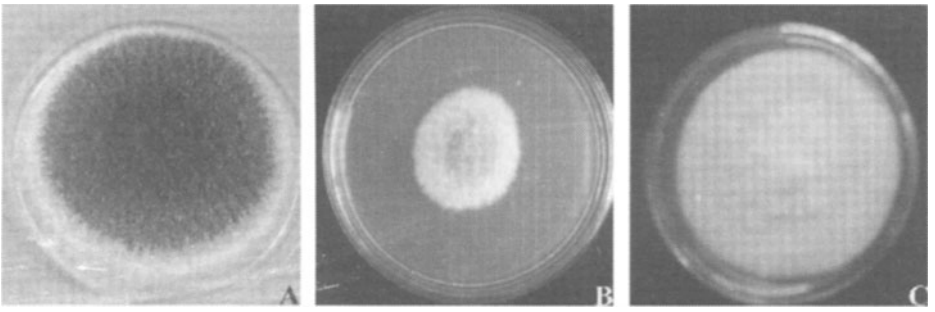

Fig. 16. 3. Growth on PDA: (A) *A. flavus* (B) *Fusarium oxysporum* and (C) *F. semitectum*

Biological synthesis of silver nanoparticles using *Aspergillus flavus* was studied by Vigneshwaran *et al.* (2007). The particles were analyzed by UV-Vis (Specord 50 ANALYTIKJENA® Spectrophotometer), by XRD (Philips® automatic X-ray Diffractometer with Philips® PW 1830 X-ray generator), by SEM (Philips® XL 30 SEM at 12–15 kV), by TEM (Philips® EM208 operating at 200 kV), FTIR (IRPrestige-21® Fourier Transform Infrared Spectrophotometer) and by photoluminescence spectra (Perkin Elmer LS55® Spectrofluorimeter using 90 illumination).

A characteristic surface plasmon absorption band at 420 nm was observed after 24 hours of reaction with maximum intensity in 72 hours. XRD analysis showed the planes of the face-centered cubic (FCC) silver (planes : 111, 200 and 220) referring to peaks in 38.5°, 44° and 64.5° in spectrum of 2θ, respectively. The XRD analysis of mycelium after exposure with silver ions exhibit more peaks due to the interaction of silver nitrate with fungal cell wall 2 matrix. Furthermore, broadening peaks was observed due to the small particle size of silver. The lattice constant calculated from this pattern was 4.087 Å, a value similar to the literature report. Isotropic nanoparticles (i.e., low aspect ratio) in shape and reasonably monodisperse were observed by TEM. Size particles, measured by TEM, were 8.92±1.61 nm and any agglomeration was observed. The SEM technique was used to characterize the fungal mycelium challenged with silver nitrate solution. SEM micrograph showed silver nanoparticles deposited in the fungal mycelium. The diffraction pattern confirmed the face-centered cubic 'FCC' crystalline structure of metallic silver.

The FTIR analysis confirmed the presence of protein in the samples of silver nanoparticles. An emission peak with very broad base in 523 nm was observed in photoluminescence spectra as fungal protein bound to silver nanoparticles.

Fusarium species

Fusarium is one of the most important genera of plant pathogenic fungi and widely distributed on plants and in soil. It also causes infection in animals and human beings. *Fusarium* species are responsible for wilts, blights, root-rots, and cankers in coffee, pine trees, wheat, corn, rice, carnations and grasses leading to the great economic losses (Hocking, 1987; Knoll *et al.*, 2002; Miller, 2002). *Fusarium* sp. grow rapidly on simple media like Potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA) at 25°C and produce woolly to cottony, flat, spreading colonies. From the front, the colour of the colony may be white, cream, tan, salmon, cinnamon, yellow, red, violet, pink, or purple. From the reverse, it may be colorless, tan, red, dark purple, or brown. Microscopically *Fusarium* can be easily identified by its curve shaped and septate macroconidia which is its important characteristics. While hyaline septate hyphae, conidiophores, phialides, and microconidia are observed microscopically. In addition to these basic elements, chlamydospores are also produced in some species (Seifert, 1996).

Fusarium species have been extensively studied for their potential towards the synthesis of metal nanoparticles and therefore their morphology has been discussed in detail in this chapter.

Fusarium oxysporum

Fusarium oxysporum is the most widely dispersed of the *Fusarium* species and can be recovered from most soil of the regions and also found in association with many important crop plants. It causes *Fusarium* wilt disease in more than a hundred species of plants. It does so by colonizing the water-conducting vessels

(xylem) of the plant. As a result of this blockage and breakdown of xylem, symptoms appear in plants such as leaf wilting, yellowing and eventually plant death. Most commonly it is the causative agent of Panama disease of Banana (Fig. 16.3 B).

Morphologically the colonies of *F. oxysporum* on PDA varies widely. Mycelia may be floccose, sparse or abundant and range in color from white to pale violet. Abundant pale-orange or pale-violet macroconidia are produced in a central spore mass in some isolates. Small pale-brown, blue to blue-black or violet sclerotia may be produced abundantly by some other isolates. *F. oxysporum* usually produces a pale to dark-violet or dark-magenta pigments in the agar but some isolates produce no pigment at all. Some isolates of *F. oxysporum* mutate readily to the pionnotal form or to a flat "wet" mycelial colony with a yellow to orange appearance when cultured on PDA (Seifert, 1996).

The critical morphological features of *F. oxysporum* include the production of microconidia in false heads on short phialides formed on the hyphae, the production of chlamydospores and the shape of the macroconidia and the microconidia (Seifert, 1996).

The production of silver nanoparticles by *Fusarium oxysporum* was studied by UV-Vis spectroscopy (Shimadzu dual-beam spectrophotometer-model UV-1601 PC (Ahmad *et al.*, 2003; Senapati *et al.*, 2004) showed a strong surface plasmon resonance centered at ca. 413 nm.. Absorption band in low wavelength (ca. 270 nm) was observed and it was attributed to aromatic amino acids of proteins (tryptophan and tyrosine residues). This band indicates the release of proteins into solution by *F. oxysporum* and suggested the influence of protein in silver ions reduction (Durán *et al.*, 2005).

Through fluorescence spectroscopy (Perkin-Elmer LS 50B luminescence spectrophotometer) showed an emission band centered at ca. 340 nm indicating that the proteins bound to the nanoparticle and those present in the solution exist in the native form. This result was confirmed by FTIR (Shimadzu FTIR-8201 PC instrument) by the presence of three bands at 1650 cm^{-1}, 1540 cm^{-1} and 1450 cm^{-1}. The bands at 1650 and 1540 cm^{-1} referring to amide I and II of protein indicate that the secondary structure of the proteins was not affected in the silver ions reduction process. The silver nanoparticles observed by TEM (JEOL 1200EX instrument at a voltage of 80 kV) showed individual and aggregated silver particles. The particle morphology was spherical and occasionally triangular one with size of 5-50 nm. Crystalline silver particles were obtained as verified by diffraction pattern (X-ray diffraction-XRD), which was carried out in the transmission mode on a Philips PW 1830 instrument operating at 40 kV. The particle size measured by XRD using Debye-Scherrer's equation 7 nm. Similar results were obtained by Durán *et al.* (2005). In this work silver nanoparticles produced by *F. oxysporum* exhibited plasmon absorbance at 420 nm in the UV-Vis analysis (Agilent 8453—diode array) and size, measured by SEM (Jeol – JSM-6360LV), of 20-50 nm. TEM (Carl Zeiss CEM-902 transmission electron

microscope at 80 KeV) images of particles produced by the same fungus showed spherical particles with size of 1.6 nm calculated by XRD (XRD, model XD3A from Shimadzu) and the Scherrer's equation (Durán *et al.*, 2007). These nanoparticles were analyzed by elemental spectroscopy imaging (ESI) showing that the nanoparticles were formed by silver and the presence of the N and S atoms around the silver nanoparticles. This result was associated with the particle stabilization by the fungal proteins.

Fusarium semitectum

Fusarium semitectum is commonly isolated from soil and from diverse aerial plant parts in tropical and sub-tropical areas, *e.g.*, banana fruits and palm fronds, but it can also be recovered from soils in the arctic and deserts. Although there are many reports of *F. semitectum* being implicated in various diseases it is often not regarded as an important plant pathogen. It has been reported to cause a canker of walnut, pod and seed-rot of beans, reduced seed germination and seedling growth of sorghum, corky-dry-rot of melons, and storage rot problems of mushrooms (Seifert, 1996) .

Cultures usually grow rapidly and produce abundant dense aerial mycelia which are initially off-white and become reddish or brown with age (Fig. 16.3 C). Brown pigments may also be produced in the agar. Light-orange sporodochia may be produced by some strains.

The silver nanoparticles were synthesized by *F. semitectum* (Basavaraja *et al.*, 2008) and this production was followed by UV–Vis spectroscopy measurements (Elico spectrophotometer). The particles were characterized by XRD measurements (Siemens X-ray diffractometer (Japan)), TEM (Technai-20 Philips transmission electron microscope operated at 190 keV), and FTIR (Perkin-Elmer FT-IR Spectrum ONE).

The UV-Vis analysis confirmed the silver nanoparticles production by the surface plasmon resonance band at 420 nm. Silver nanoparticles formation was also confirmed by XRD, diffraction signals to corresponding facets of silver (1 1 1, 2 0 0, 2 2 0 and 3 1 1). The average particles size was calculated by XRD using the diffraction signal in 1 1 1 plane and the Scherrer's equation giving 35 nm. These particles were analyzed by TEM showing particles polydisperse and in spherical shape. From the FTIR analysis it was observed that the dispersion in the protein is due to the presence of bands referring to amide I and amide II and linkages of the proteins.

Fusarium acuminatum

Fusarium acuminatum found in temperate regions usually as a soil saprophyte or associated with roots and crowns of plants. It can occasionally be associated with root and crown diseases of a variety of hosts, especially legumes, and has been recovered from cereal grains in Canada, Europe, India and the

former Soviet Union. Recently, it has been reported that *F. acuminatum* can produce traces of mycotoxins like trichothecenes.

It is a relatively slow-growing species that produces white mycelium which is abundant in some isolates (Fig. 16.4 A). The mycelium is floccose with rose to burgundy pigmentation that can be grayish rose at the periphery. Sporodochia formed in the center of the colony in a small central spore mass and are pale-orange to dark brown. Red pigments (sometimes brown) are formed in the agar (Seifert, 1996).

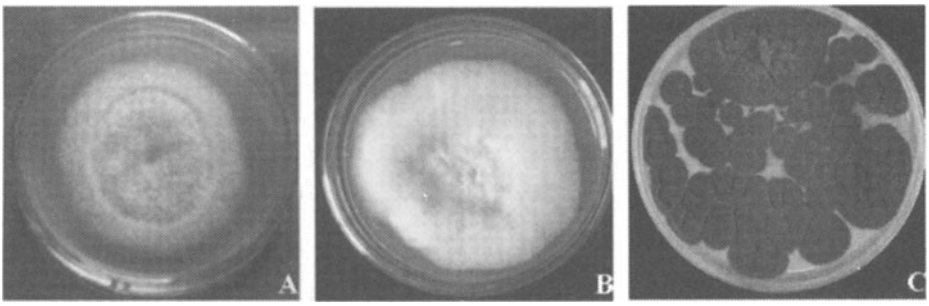

Fig. 16.4. Growth on PDA: (A) *F. acuminatum* (B) *F. solani* and (C) *Penicillium* sp.

Extracellular mycosynthesis of silver nanoparticles by *Fusarium acuminatum* Ell. and Ev. was reported in the literature (Ingle *et al.*, 2008). Plasmon resonance band was observed at 420 nm in the UV-Vis spetrum Perkin-Elmer - lambda-25). After 15-20 minutes of reaction with silver ions the dispersion color changed from yellowish to brown indicating the silver nanoparticles formation. These particles were analyzed by TEM (Phillips, CM12) operating at 120KV. TEM images showed spherical and polydisperse particles in the range of 5-40 nm. The particle size distribution histogram determined by TEM showed the large variation in the particle size with about 30% of the particles in the range of 0-5 nm and 7% in 40-45 nm ranges.

Fusarium solani

Fusarium solani is commonly distributed in soil and on plants. It causes onion-rot disease and also found associated with other crop plants and food grains. *F. solani* is, however, well documented as a pathogen of a number of legumes and other tropical plants where it is often associated with cankers and die-back problems of trees. Some economically important plants with significant diseases caused by *F. solani* includes, beans, citrus, cowpea, orchids, peas, peppers and potato.

It is most resistant species to the commercially available antifungal agent. With respect to human pathogenicity, *F. solani* has been recovered from eyes, nails and skin, bone, nasal cavities, infected wounds, and systemically infected cancer and HIV patients. Patients with keratitis resulting from infection with *F.*

solani were more likely to be HIV positive. *F. solani* may also cause endocarditis and lung disease, and has been shown to be allergenic (Seifert, 1996).

Cultures of *F. solani* usually are white to cream with sparse mycelium (Fig. 16.4 B). Sporodochia often are produced in abundance and may be cream, blue or green. Many isolates do not produce pigments in the agar although some violet or brown pigments may be observed.

F. solani isolated from infected onion was used for the biosynthesis of silver nanoparticles (Ingle *et al.*; 2009), silver nanoparticles produced were characterized by UV-Vis spectrophotometer (Perkin-Elmer-lambda-25), the UV-Vis spectrum showed a absorption peak at 420 nm and the FTIR (Perkin-Elmer) analysis carried out provides the evidence for the presence of proteins which helps for the stabilization by capping to the nanoparticles. TEM (Phillips, CM12) micrographs showed the production of spherical nanoparticles in the range of 5-35 nm with average size diameter of 16.32 nm.

Penicillium sp.

Penicillium is a mold that is widely distributed in nature, and is often found living on food and in indoor environments (Fig. 16.4C). The main species of *Penicillium* are *P. chrysogenum* and *P. notatum* (Samson *et al.*, 1977). It has rarely been reported as a cause of human disease. It is the source of several β-lactam antibiotics, most significantly penicillin. Other secondary metabolites of *P. chrysogenum* include various different penicillins, roquefortine C, meleagrin, chrysogine, xanthocillins, secalonic acids, sorrentanone, sorbicillin, and PR-toxin (de Hoog *et al.*, 2000).

Like many other species of the genus *Penicillium*, *P. chrysogenum* reproduces by forming dry chains of spores (or conidia) from brush-shaped conidiophores. The conidia are typically carried by air currents to new colonisation sites. In *P. chrysogenum* the conidia are blue to blue-green, and the mold sometimes exudes a yellow pigment. However, *P. chrysogenum* cannot be identified based on colour alone. Observations of morphology and microscopic features are needed to confirm its identity.

The airborne spores of *P. chrysogenum* are important human allergens. Vacuolar and alkaline serine proteases have been implicated as the major allergenic proteins (Shen *et al.*, 2003). *P. chrysogenum* has been used industrially to produce penicillin and xanthocillin X, to treat pulp mill waste, and to produce the enzymes polyamine oxidase, phospho-gluconate dehydrogenase, and glucose oxidase (de Hoog *et al.*, 2000; Shen *et al.*, 2003).

Penicillium strain isolated from soil (Sadowski *et al.*, 2008) produced silver nanoparticles which were monitored by UV-Visible spectrophotometer (HELIOS λ, ThermoElectron Corp.). The particles size was measured by laser diffractometry using a Mastersizer 2000 instrument (Malvern) equipped with HydroMu dispersing unit (Malvern) and the zeta potential was carried out using a Zetasizer Nano ZS (Malvern) and a titrator MPT-2.

Two bands were observed in the UV-Vis spectrum. The first one was a shoulder at 370 nm and the second was a band at 440 nm corresponding to excitation of transversal and longitudinal plasmon vibration in silver nanoparticles, respectively. Polydispersity of particles was observed by laser diffraction. The size was in the range of 100 nm until micrometers. This result was confirmed by SEM. The SEM micrograph of silver nanoparticles dried showed particles agglomerated. This polydispersity can be due to the drying process made before analysis in laser diffraction and SEM. The nanoparticles obtained exhibit negative zeta potential and this potential changed drastically from pH 2 to pH 8 presenting isoelectric point in pH below 2. In acid pH the particles were instable but in pH > 8 the particles were stable due to the electrostatic repulsion.

Trichoderma asperellum

Trichoderma species are present in soil, where they are the most prevalent culturable fungi. Many species in this genus can be characterized as opportunistic avirulent plant symbionts (Harman *et al.*, 2004).

Cultures are typically fast growing at 25-30°C, but do not grow above 35°C. Colonies at first appear transparent on media such as cornmeal dextrose agar (CMD) or white on richer media such as potato dextrose agar (PDA). Mycelium typically not obvious on CMD, conidia typically forming within one week in compact or loose tufts in shades of green or yellow or less frequently white. Yellow pigment may be secreted into the agar, especially on PDA. A characteristic sweet or 'coconut' odor is produced by some species (Harman *et al.*, 2004).

Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of plants. The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, and competition. Most biocontrol agents are from the species *T. harzianum, T. viride*, *T. hamatum* and *T. asperellum*. The biocontrol agent generally grows in its natural habitat on the root surface, and so affects root disease in particular, but can also be effective against foliar diseases (Harman, 2006).

Trichoderma asperellum were used to produce the silver nanoparticles (Mukherjee *et al.*, 2008). The bioproduction was detected by UV-Vis spectrophotometer (JASCO double-beam UV–Vis spectrophotometer model V-530); the particles were characterized by XRD (Philips X'pert Pro XRD unit); by Transmission electron microscopy and selective area electron diffraction (SAED) (JEOL 2000 .FX machine); by FTIR at room temperature (JASCO FTIR spectrometer model No. 4100). Furthermore, the silver nanoparticles were analyzed by macro-Raman set-up using 532 nm line of a diode laser for excitation, 0.9 m single-stage monochromator to the scattered light and a CCD detector. The particle size was measured by dynamic light-scattering (DLS) using 532 nm line of a He–Ne laser as the source for excitation.

The silver nanoparticles exhibited an intense peak at 410 nm corresponding to the surface plasmon resonance. The particles size, calculated by XRD applying Scherrer's equation was 17 nm and the XRD pattern exhibited a broad and intense peak at 38.4° and a hump at 64.5°. These peaks corresponding to diffraction from the (111) and (220) planes of silver with "FCC" lattice. The broadening of the peaks indicates that the particles are in the nanometric range. This result was confirmed by selected-area electron diffraction pattern. In this analysis was observed concentric rings referring to (111), (311) and (220) planes of silver. The particles size, measured by TEM, was in the range 13–18 nm.

After 6 month, the silver nanoparticles size was measured by DLS showing 35.4 nm. This value is larger than size measured by XRD and TEM because in DLS analysis is considerate in the size of the hydrated capping agents (proteins) and the solvation effects. Thus, the hydrodynamic diameter determinated by DLS (could be as high as 1.2 times the original diameter of the capped particles).

FTIR spectroscopic studies together with Raman spectrum gave the data to establish the silver nanoparticles biosynthetic mechanism and the nature of the capping species identified. Raman analysis showed selective enhancement of certain chemical bond vibrations in organic moieties (the capping agents) associated with the silver particles that extends the feasibility of using these nanoparticles as potential templates for surface enhanced resonance Raman spectroscopy (SERS).

Coriolus versicolor

A controlled and up-scalable method to produce silver nanoparticles mediated by *Coriolus versicolor* was published (Sanghi and Verma, 2009). The nanoparticles were characterized by UV/Vis Spectrophotometer (Perkin Elmer, USA), Infrared (IR) using reflectance mode (BRUCKER, VERTEX-70), XRD (ARL X TRA X-ray Diffractometer). The fungal mycelium with and without silver nanoparticles was dried and after analyzed by XRD. The particles were also characterized by TEM (Philips - EM208 operating at 200 kV) and Atomic force microscopy (AFM) (PicoscanTM Molecular imaging, USA).

The absorption spectrum showed the band centered at 430 nm and this band was blue shifted to 420 nm when the pH was changed to 10 indicating the decrease particle size. By the XRD analysis was observed the diffraction peaks corresponding to facets of silver (111, 200, 311) indicating the formation of crystalline silver nanoparticles. Furthermore, the XRD pattern of fungal mycelium after exposition with silver ions showed more diffraction peaks referring to Ag_2S ($2\theta = 33.3°$, $39.2°$, $46.2°$), Ag_3O_4 ($2\theta = 32.09°$), AgO ($2\theta = 46.0°$) and peaks corresponding to silver ions ($2\theta = 55°$, $65.5°$, $68.8°$).

FTIR gave information regarding silver nanoparticles formation mechanism and composition of thiol derivatives surrounding the metallic cores. After silver nanoparticles produced was observed a new band in 1735 cm^{-1} referring to carbonyl stretch vibrations in ketones, aldehydes and carboxylic acids. This result

indicates that the reduction of the silver ions is coupled to the oxidation of the hydroxyl groups in fungal mycelium molecular and/or its hydrolysates. Also was observed in the FTIR spectrum the ν SH stretches (2590–2540 cm^{-1}). After the reaction, at alkaline pH, the band at 2526 cm^{-1} completely disappears indicating the formation of a bond between the S atoms and silver clusters (Ag-S bond). Silver nanoparticles spherical and symmetrical with size of 10 nm and without aggregation were observed by TEM. Besides, by TEM, agglomerated particles were observed when the pH was changed showing the influence of pH in the silver nanoparticles formation.

Cladosporium cladosporioides

Extracellular biosynthesis of silver nanoparticles was studied using the fungus *Cladosporium cladosporioides* (Balaji *et al.*, 2009). Silver nanoparticles were analyzed by UV-Vis, TEM (Technai-20 Philips transmission electron microscope operating at 190 keV), XRD (Siemens X-ray diffractometer), FTIR (PerkinElmer FT-IR Spectrum ONE at a resolution of 4 cm^{-1}).

UV–Vis spectrum showed the band corresponding to the surface plasmon resonance at 415 nm indicating the silver nanoparticles formation. Besides, size, calculated by Scherrer's equation was 35 nm. This value was in agreement with TEM results. The TEM image showed polydisperse and spherical particles with size in the range of 10-100 nm. The bands corresponding to amide I and amide II from proteins was identified in the FTIR spectrum confirmed the presence of protein that can bind to silver nanoparticles stabilizing these particles in the medium.

Multiple applications of metal nanoparticles

Metal nanoparticles have many physicochemical and optoelectronic properties. Due to all diverse properties, nanoparticles have multiple applications in various fields like electronics, agriculture and medicine. Some of the nanoparticles that can be produced by fungi may be of particular relevance to new and emerging technologies. The use of silver coatings in solar absorption systems has already been mentioned. Other applications in such areas include the use of gold nanoparticles as precursors to coatings for electronic applications (Mukherjee *et al.*, 2001a) and platinum nanoparticles in the production of fuel cells (Riddin *et al.*, 2006).

The application in the field of medicine includes the formulations of many potential antimicrobial agents, which are effective against many human pathogens including multidrug-resistant bacteria (Ingle *et al.*, 2008). Ingle *et al.* (2008) evaluated the biosynthesized silver nanoparticles from *F. acuminatum* for their broad spectrum antibacterial activity on different human pathogens. The authors reported efficient antibacterial activity of silver nanoparticles against multidrug resistant and highly pathogenic bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhi* and *Escherichia coli*.

Silver nanoparticles showed large antimicrobial effect than silver ions (1.4-1.9 folds). The maximum antibacterial activity of silver nanoparticles was against *Staphylococcus aureus* (17 mm), followed by *Staphylococcus epidermidis*, *Salmonella typhi* and the minimum by *E. coli* (10 mm). This result demonstrated that specific efficiency of silver nanoparticles can be related with differences from strain, which can be due the bacterial membrane structure. However, it warrants further investigation.

Sondi and Sondi (2004) reported antimicrobial activity of silver nanoparticles against *E. coli* as a model for Gram-negative bacteria. From the SEM micrographs, formation of aggregates composed of silver nanoparticles and dead bacterial cells were observed. It was also observed that the silver nanoparticles interact with the building elements of the bacterial membrane and cause damage to the cell. The TEM analysis and EDAX study confirmed the incorporation of silver nanoparticles into the membrane, which was recognized by formation of pits on the cell surface. They concluded that nanomaterials could prove to be simple, cost effective and suitable for formulation of new type of bacterial materials.

Another application of silver nanoparticles is the production of sterile materials. Cotton fabrics incorporated with 2% of silver nanoparticles produced by *F. oxysporum* (Durán *et al.*, 2007), exhibited high antibacterial effect against *S. aureus* (99.9% bacterial reduction). The fabrics, after the antibacterial assay, were analyzed by SEM-EDS showing the presence of the silver peak and the absence of the contamination with bacteria. These results demonstrated that silver nanoparticles can be used to turn sterile fabrics. Furthermore, the silver nanoparticles dispersion will be reused for impregnation of other fabrics, for example, working in a closed circuit causing less damage to the environment.

Kim *et al.* (2007) investigated the antimicrobial activity of silver nanoparticles against *E. coli* and *S. aureus* on Mullar Hinton agar plates. In this study yeast and *E. coli* were inhibited at low concentration of silver nanoparticles, where as the growth inhibitory effects on *S. aureus* were mild. Shahverdi *et al.* (2008) studied the activity of silver nanoparticles against *E. coli* and *Staphylococcus aureus*. Duran *et al.* (2007) used the silver nanoparticles in the preparations of cotton fabrics and also evaluated its activity against *S. aureus*. Rai *et al.* (2009) suggested that the silver nanoparticles produced by the fungi are the novel and new generation of antimicrobials.

Edelstein *et al.* (2000) developed the Bead ARray Counter (BARC) as a multi-analyte biosensor from nanoparticles which are used in DNA hybridization, magnetic microbeads, and giant magnetoresistive (GMR) sensors for the detection and identification of biological warfare agents. De La Isla *et al.* (2003), cover teeth surfaces with nanohybrid coatings containing an inorganic ceramic and an organic copolymer constituents. They reported the first ever values of scratch penetration depth and scratch recovery for bare and coated teeth. The authors found that uncoated teeth undergo viscoelastic recovery (healing) after microscratching, the first manifestation of bone viscoelasticity in tribology. The

coatings fill "valleys" in teeth surfaces. They concluded that improvement in the scratch resistance increases as compared to uncoated teeth is seen.

Cai *et al.* (2005) reported the nanotube spearing approach, in which they used nickel embedded carbon nanotubes coated with DNA. They further reported that when the nanotubes were introduced in the cells in presence of specially oriented magnetic field, the nanotubes align with the magnetic flux lines as they are pulled towards the cells. This enables the nanotubes to spear the cells, pass through the membrane and deliver the targeted DNA.

Final remarks

The most important methodology for silver nanoparticles synthesized by fungi was discussed and from this information it was possible to deduce that the control size and reactivity of silver nanoparticles caped with protein are quite different. The characterization of silver nanoparticles is very important to understand the mechanism of formation of these particles and its application. Several techniques can be used to analyze these particles such as UV-Vis spectroscopy, microscopy techniques, diffraction X-ray, fourier-transformed-infrared spectroscopy, fluorescence and photoluminescence. The reports of production of the nanoparticles are numerous, but the applications are still under study. The importance of protein on the surface of silver nanoparticles is still an open question. Further, it is also important to investigate whether different fungi have different proteins or not?.

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